American Journal of

Veterinary Research

CONTENTS

The Pathology of Total Body Radiation in Dogs Which Died Following Exposure to a Lethal Dose 329
C. A. Gleiser

Clinical Electrocardiograms of the Dog 336
O. A. Soave

Metopon Analgesia in the Dog 338
G. P. Epling and A. D. Rankin

Electrophoretic Study of the Horse Serum of Equine Infectious Anemia 343
K. Y. T. Kao, R. L. Reagan, and A. L. Brueckner

Research on Foot-and-Mouth Disease. VII. Cultivation of the Virus on a Practical Scale in Explantations of Tongue Epithelium. A Modification of the Culture Technique 346
H. S. Frenkel

The Effects of Some Phenothiazine Derivatives and Analogs on the Free-Living Stages of Horse Strongyles 349
N. D. Levine and V. Ivens

The Negative Role of the Ewe in the Transmission of Vibriosis of Sheep 352
H. Marsh, B. F. Firehammer, and L. H. Scrivner

Preliminary Studies of Parasitism in Sheep on Rangelands 356
N. F. Baker, W. M. Longhurst, D. T. Torell, and W. C. Weir

A Comparison of *Clostridium Perfringens Epsilon* Toxin-Toxoid and Prototoxin-Toxoid in the Immunization of Young Lambs 361
L. DS. Smith and T. Matsuoka

Transmissible Gastroenteritis in Pigs 364
K. M. Lee, M. Moro, and J. A. Baker

Experimental Tocopherol Deficiency in Young Calves 373
J. W. Safford, K. F. Swingle, and H. Marsh

The Teeth of the Ox in Clinical Diagnosis. II. Gross Anatomy and Physiology 385
N. L. Garlick

A Cytological Study of Costal Marrow. II. The Adult Cow 395
M. L. Calhoun

"Deperitonealization" of the Wall of the Rumen 405
E. F. Ebert, B. B. Roseboom, and H. E. Dale

(Continued on page 337)

A Publication of the American Veterinary Medical Association

Volume XV JULY, 1954 No. 56

Instructions to Authors

AMERICAN JOURNAL OF VETERINARY RESEARCH

Exclusive Publication.—Articles submitted for publication are accepted with the understanding that they are not submitted to other journals, which is ethical publication procedure.

Manuscripts.—Manuscripts, including **footnotes, references,** and **tables,** must be typewritten, **double-spaced,** on 8½- by 11-in. **bond paper,** and the **original,** not the carbon copy, submitted. One-inch margins should be allowed on the sides, with 2 in. at top and bottom. Articles should be concise and to the point. Short, simple sentences are clearer and more forceful than long, complex ones.

Illustrations.—Photographs should be furnished in glossy prints, and of a size that will fit into the AMERICAN JOURNAL OF VETERINARY RESEARCH with a minimum of reduction. Photomicrographs which can not be reduced should be marked for cropping to 1-column or 2-column width. Identifying marks within the photomicrographs, such as arrows, letters, or numbers, should be clearly marked with black India ink or white opaque ink to insure good reproduction and must be large enough to stand reduction, if necessary. Drawings, graphs, and charts should be made clearly and accurately in India ink on white paper and a glossy print of them submitted when possible. Numbers or letters appearing on graphs or charts should be large enough to allow for any reduction necessary for the chart or graph to fit Journal pages. Blue lines in graph paper drop out in reproduction; therefore, if lines are required they must be drawn in black ink. All illustrations should bear the name of the author and the illustration number on the back.

Tables.—Tables should be simple and typed **double space.** Complex tables are not conducive to perusal. It is wiser to summarize complex material than to tabulate it.

References.—References should be typed **double space** and should be prepared in the following style: name of author, title of article, name of periodical with volume, year, and page numbers. References to journals not commonly known should give the complete name of the periodical, and where published so that they may be added to our reference files. When books are cited, the name of publisher, location, edition, and year should be given.

Editors

L. A. MERILLAT (editor emeritus), W. A. AITKEN, J. G. HARDENBERGH, HELEN S. BAYLESS

Committee on Journal: EDWIN LAITINEN, JAMES A. MCCALLAM, A. H. QUIN.

Research Council

Anatomy and Histology

ROBERT GETTY, D.V.M., Ph.D. *(Secretary)*, Professor of Veterinary Anatomy and Histology, Division of Veterinary Medicine, Iowa State College, Ames, Iowa (1955).

Bacteriology (Immunology and Biological Therapy)

L. C. FERGUSON, D.V.M., Ph.D. *(Chairman)*, Department of Veterinary Science, Ohio Agricultural Experiment Station, Wooster, Ohio (1956).

Biochemistry and Animal Nutrition

M. J. SWENSON, D.V.M., Ph.D., Department of Physiology, School of Veterinary Medicine, Kansas State College, Manhattan, Kan. (1956).

Large Animal Medicine

E. F. EBERT, D.V.M., Head, Department of Medicine and Surgery, School of Veterinary Medicine, University of Missouri, Columbia, Mo. (1956).

Large Animal Surgery

A. G. DANKS, B.S., D.V.M., Professor, Department of Surgery, New York State Veterinary College, Cornell University, Ithaca, N. Y. (1954).

Parasitology

R. D. TURK, D.V.M., Head, Department of Veterinary Parasitology, School of Veterinary Medicine, Texas A. & M. College, College Station, Texas (1954).

Pathology

T. LLOYD JONES, D.V.M., M.Sc., Principal, Ontario Veterinary College, Guelph, Ont. (1956).

Physiology and Pharmacology

D. K. DETWEILER, V.M.D., M.S., Associate Professor of Pharmacology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pa. (1956).

Poultry Pathology

C. A. BRANDLY, D.V.M., M.S., Chairman, Department of Veterinary Science, University of Wisconsin, Madison, Wis. (1954).

Radiology

M. A. EMMERSON, D.V.M., M.S., Dr. Med. vet. (Zurich), Professor of Veterinary Obstetrics, Division of Veterinary Medicine, Iowa State College, Ames, Iowa (1954).

Small Animal Medicine

J. C. FLINT, D.V.M., M.S., Practitioner, 1220 S. State St., Salt Lake City, Utah (1955).

Small Animal Surgery

J. E. GREENE, D.V.M., M.S., Professor of Surgery and Medicine, College of Veterinary Medicine, Alabama Polytechnic Institute, Auburn, Ala. (1955).

Veterinary Hygiene

ALFRED G. KARLSON, D.V.M., Ph.D., Associate in Experimental Pathology, Mayo Foundation, Rochester, Minn. (1955).

Virus Diseases

C. H. CUNNINGHAM, D.V.M., Ph.D., Associate Professor of Bacteriology and Public Health, School of Veterinary Medicine, Michigan State College, East Lansing, Mich. (1955).

Member-at-Large

D. A. SANDERS, D.V.M., Head, Department of Veterinary Science, University of Florida, Gainesville, Fla. (1954).

Published quarterly at 600 S. Michigan Ave., Chicago, Ill., by the American Veterinary Medical Association. Entered as Second-Class Matter January 31, 1941, at the Post Office at Chicago, Illinois, under the Act of March 3, 1879. Additional entry as Second-Class Matter at the Post Office at Champaign, Illinois, approved February 17, 1953.

Reproduction of any part of this publication is prohibited, unless special permission is given by the publishers and the authors.

Subscription price: $5.00 per year for United States and countries affiliated with the Pan American Postal Union; Canada and foreign countries, $5.30. Members of the American Veterinary Medical Association may subscribe to the American Journal of Veterinary Research for $2.50 per year added to the annual dues of $15.00. Single copies $1.25. Address all correspondence to American Veterinary Medical Association.

(Contents continued from cover)

A Comparison of the Effects of Subcutaneous and Intracaudal Vaccination of Sexually Mature Cattle with *Brucella Abortus* Strain 19 406
D. T. Berman, B. A. Beach, and M. R. Irwin

Bovine Blood Serum Concentrations of Terramycin (Oxytetracycline) Following Intravenous and Intramuscular Administration 412
H. W. Yoder, Jr., and R. A. Packer

New Studies on Comparative Brucella Immunity with Agglutinogenic and Nonagglutinogenic Vaccines 417
F. Sieiro

Rumenitis and Its Relation to Rate of Change of Ration and the Proportion of Concentrate in the Ration of Cattle 425
R. Jensen, W. E. Connell, and A. W. Deem

Experimental Infection of Calves with *Strongyloides Papillosus* (Nematoda) 429
H. H. Vegors

Application of the Capillary Tube Test and a Newly Developed Plate Test to the Serodiagnosis of Bovine Leptospirosis 434
H. G. Stoenner

Studies on *Neoascaris Vitulorum*. II. The Resistance of the Ova to Certain Chemical Agents and Physical Factors Under Tropical Conditions 440
P. G. Refuerzo and F. S. Albis-Jimenez

The Liver of the Camel as Revealed by Macroscopic and Microscopic Examinations 444
A. E. H. Hegazi

A Brief Histology of the Intestine of the Turkey Poult 447
D. D. Demke

The Morphogenesis of the Testes of Chicken Embryos 450
W. G. Venzke

The Effects of Dietary Deficiencies of Vitamin E and the B Complex Vitamins on the Electrocardiogram of Chickens 457
P. D. Sturkie, E. P. Singsen, L. D. Matterson, A. Kozeff, and E. L. Jungherr

A Preliminary Report of Efficiency of Various Mediums for Isolation of Pleuropneumonia-Like Organisms from Exudates of Birds with Chronic Respiratory Disease 463
H. E. Adler, Y. Yamamoto, and R. A. Bankowski

Studies of Ntaya, Ilhéus, and Bunyamwera Viruses in Young Dogs 466
R. L. Reagan, E. C. Delaha, M. T. Stewart, and A. L. Brueckner

The Detoxification of Chloral Hydrate 468
L. C. Payne

The Differential Effect of Basic Fuchsin and Thionin on *D*-Alanine Utilization by the Genus Brucella 472
H. S. Cameron and M. E. Meyer

Assay of Antibiotics by Use of Methylene Blue Milk 475
I. A. Schipper and W. E. Petersen

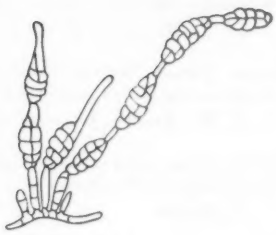
Susceptibility and Serological Response of Various Species of Animals to Infection with the Virus of Vesicular Stomatitis 477
T. Kowalczyk and C. A. Brandly

Response of the Green Turtle *(Pseudemys Elegans)* to Several Virus Strains after Intracerebral Inoculation 481
R. L. Reagan, E. C. Delaha, M. T. Stewart, and A. L. Brueckner

DIFCO

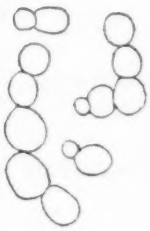
Media for

MYCOLOGY



DIAGNOSTIC

. . . for the isolation, identification and cultivation of pathogenic fungi. These media are also widely used in phytopathological studies. Several are neutral in reaction, giving optimum conditions for growth of a variety of fungi. The following may be prepared as selective media by the adjustment of reaction, addition of antibiotics or other agents:



Bacto-Brain Heart Infusion Agar
Bacto-Sabouraud Dextrose Agar
Bacto-Sabouraud Maltose Agar
Bacto-Littman Oxgall Agar
Bacto-Mycological Agar
Bacto-Mycological Broth
Bacto-Corn Meal Agar
Bacto-Corn Meal Agar with Dextrose
Bacto-Bean Pod Agar
Bacto-Prune Agar
Bacto-Lima Bean Agar

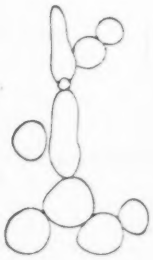
CONTROL

. . . for sanitary and sterility procedures as well as for general use in mycological procedures:

Bacto-Sabouraud Maltose Broth
Bacto-Sabouraud Liquid Medium
Bacto-Malt Extract
Bacto-Malt Agar
Bacto-Neurospora Culture Agar
Bacto-Potato Dextrose Agar
Bacto-Mildew Test Medium
Bacto-W.L. Nutrient Medium
Bacto-W.L. Differential Medium

CLASSIFICATION

. . . and nutritional studies of fungi:



Bacto-Yeast Morphology Agar
Bacto-Yeast Carbon Base
Bacto-Yeast Nitrogen Base
Bacto-Czapek Dox Broth
Bacto-Czapek Solution Agar
Bacto-Vitamin Free Yeast Base

THE DIFCO MANUAL, *NINTH EDITION,*
including descriptions of these media and their use, is available on request.

DIFCO LABORATORIES
DETROIT 1, MICHIGAN

American Journal of Veterinary Research

COPYRIGHT 1954 BY AMERICAN VETERINARY MEDICAL ASSOCIATION

VOL. XV JULY, 1954 No. 56

The Pathology of Total Body Radiation in Dogs Which Died Following Exposure to a Lethal Dose

Lieutenant Colonel CHESTER A. GLEISER, V.C., U.S.A.

Washington, D. C.

THE SEQUENCE of the early pathological changes in dogs exposed to $l.d._{100/30}$ of total body x radiation has been described in a previous communication.[1]

The purpose of this paper is to report the gross and microscopic changes observed at necropsy in dogs that had received 600 r of total body radiation. Six hundred roentgens is considered $l.d._{100/30}$ of total body x radiation for the dog; that is, this amount will kill all of the dogs exposed within thirty days. The range of survival time for these dogs was four to twenty-one days and the mean survival time was twelve days. The complete data on survival time have been reported.[2]

MATERIALS AND METHODS

The 2-million-volt industrial x ray machine at the Naval Ordnance Laboratory was used to irradiate the dogs. The characteristics of the machine, the technique employed, and the dogs used in the study have been described in earlier reports.[1,2]

All animals were allowed to die and were examined immediately after death, both grossly and microscopically. Specimens of rib and femur marrow were obtained. Tissues removed at necropsy were cut in thin sections and immediately fixed in 10 per cent neutral buffered formalin. Suitable tissue blocks were dehydrated and embedded in the usual manner. Sections were cut at 5 μ and stained with hematoxylin-eosin. Marrow sections were decalcified electrically and stained by Giemsa's method.

This study was performed at the Naval Medical Research Institute, National Naval Medical Center, Bethesda, Md., and the Armed Forces Institute of Pathology, Washington, D. C.

The author is deeply indebted to Brig. Gen. Elbert DeCoursey, M.C., U.S.A., director, Armed Forces Institute of Pathology, and Capt. A. W. Eaton, Jr., M.C., U.S.N., (Ret.), formerly deputy director, Armed Forces Institute of Pathology, for their guidance during this period of study. He is further indebted to Comdr. E. P. Cronkite, M.C., U.S.N., of the Naval Medical Research Institute, National Naval Medical Center, for his invaluable assistance in the irradiation of these dogs. This work could not have been accomplished without the capable assistance of the technical staffs of the Naval Medical Research Institute and the Armed Forces Institute of Pathology.

RESULTS

Clinical Symptoms.—During the first twenty-four hours after irradiation, the dogs exhibited lassitude, anorexia, and vomiting. Some of them vomited within one hour after irradiation. The rectal temperatures remained normal.

From the second day until the onset of the terminal stage, generally the eighth or ninth day after irradiation, the dogs regained appetite and appeared to be fairly well but anorexia returned and later they refused food. Rectal temperatures remained normal during this stage. During the latter part of this intermediate period, the skin of some of the dogs became ulcerated.

The onset of stomatitis frequently ushered in the terminal phase which was characterized by refusal to eat, fever, dyspnea, emaciation, bleeding gums, bloody diarrhea, subepidermal ecchymoses, conjunctival infection, and a strongly hemorrhagic reaction to minor trauma. Large subepidermal hematomas and cellulitis of one or more limbs frequently appeared before death.

Pathological Changes.—Lymphatic Tissues.—Grossly, the lymph nodes were greatly enlarged, hemorrhagic, and edematous. On cut surface, the parenchyma was soft and mushy and its architectural features were obliterated.

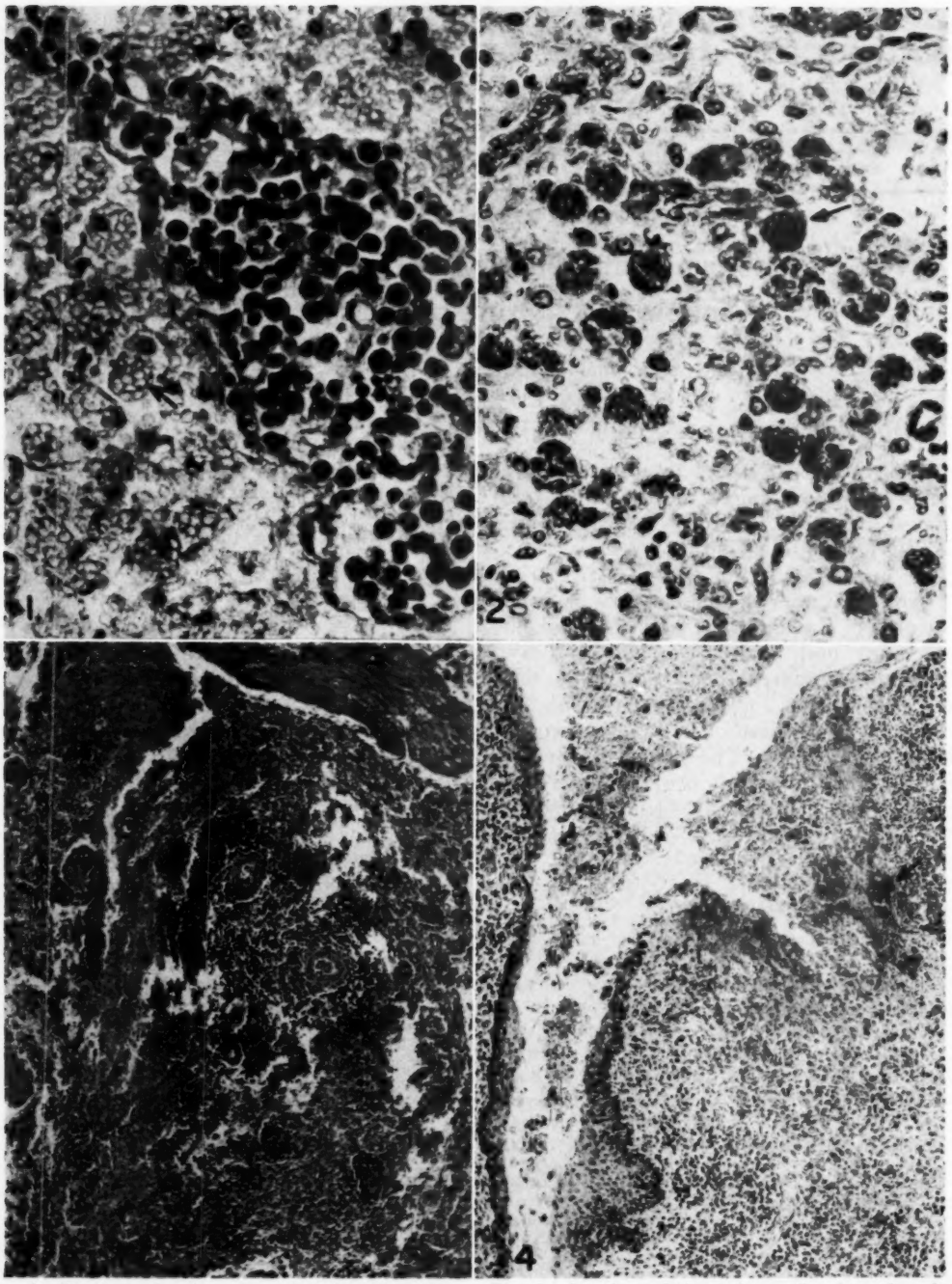


Fig. 1—Section of a lymph node of a dog nine days after receiving 600 r of total body radiation—replacement of lymphoid elements with plasma cells and massive erythrophagocytosis in sinuses. x 185. AFIP 248123.

Fig. 2—Section of a lymph node of a dog three days after receiving 900 r of total body radiation—erythrophagocytosis. x 415. AFIP 248187.

Fig. 3—Section of the spleen of a dog nine days after receiving 600 r of total body radiation—great reduction in lymphoid elements. x 70. AFIP 248123.

Fig. 4—Section of the tonsil of a dog nine days after receiving 600 r of total body radiation—ulceration and necrosis with no cellular reaction. x 90. AFIP 248123.

Microscopically, hypoplasia of the lymphoid elements was extreme with apparent survival of the reticular cells. No evidence of regeneration of the lymphoid elements was observed. Massive hemorrhage frequently obscured other changes. The medullary sinuses contained many phagocytes laden with erythrocytes, blood pigment, or cellular debris. Erythrophagocytosis was striking in many of the nodes. The same changes were apparent in other lymphatic tissues: the solitary and aggregated lymphoid follicles of the intestine, the tonsil, and the spleen. Necrosis of the tonsil in some instances was associated with abundant bacterial growth. The thymus gland was very hypoplastic.

Spleen.—Grossly, subcapsular hemorrhages of various sizes were frequent. Microscopically, hypoplasia of the lymphoid elements and apparent survival of the reticular elements of the follicles were combined with engorgement of the splenic sinuses as a result of excessive erythrophagocytosis. Numerous pigment-laden macrophages and plasma cells were observed.

Gastrointestinal Tract.—Grossly, widespread hemorrhages within the gastrointestinal tract were generally located beneath the mucosa and the peritoneum and within the muscles particularly of the large bowel but also of the small bowel and stomach. They were practically absent in the esophagus but were seen in the pharynx and oral cavity. These hemorrhages varied in size from petechiae to ecchymoses. Large hemorrhages were rarely found in these structures. Stomatitis of varying severity with and without ulceration was almost always present, accompanied by pharyngitis, often with ulceration, and necrosis. The small intestine and colon, particularly the rectum, were frequently filled with partially decomposed blood. Huge hemorrhages were numerous in the perirectal, perivesical, perirenal, and retroperitoneal areas. Similar hemorrhages were found in the mesentery of both large and small bowel. Occasionally, large dissecting hemorrhages of the stomach produced obstruction. Fibrinous exudate was occasionally seen on the peritoneal surface of the intestines. Perforation was not noted in any of the dogs examined.

Microscopically, the hemorrhages in the gastrointestinal tract split the muscle layers or elevated the peritoneum, mucosa, or both, and it was in these areas that the ulcerations were observed. In the small bowel, the ulcerations were often deep with necrotic areas extending into the muscularis; large colonies of bacteria were observed within the tissues but there was no evidence of inflammatory cellular infiltration. Degeneration and necrosis of the mucosal crypts of the small intestines were evidenced by atrophy and actual disintegration of the cells, absence of normal mitoses, and changes in staining characteristics. A few of the cells lining the villi had an increased content of mucin. The villi adjacent to the ulcers were denuded of surface epithelium. Similar ulcerations were seen within the mouth and pharynx but not in the esophagus. The region of the ileocecal valve was frequently involved in a hemorrhagic and ulcerative process.

Bone Marrow, Rib, and Femur.—Grossly, the bone marrow appeared hypoplastic to judge from its color and the deposition of fat. In some instances the marrow was hemorrhagic. Microscopic examination revealed complete suppression of all hemopoietic elements with no evidence of regeneration.

Testes.—Grossly, the testes showed varying degrees of change. The hemorrhagic tendency was manifest as petechiae, ecchymoses, or massive hemorrhages which usually were superficial and directly beneath the tunica although, occasionally, they involved the parenchyma.

Microscopically, in addition to the hemorrhages noted grossly, varying degrees of degeneration were apparent within the testicular tubules and and were seen as vacuolization, pyknosis, and necrosis within the germ cell layer of the spermatogonia and within the spermatocytes. In some cases, spermatocytes had almost completely disappeared. Degeneration was spotty and did not always involve the entire testis. Sertoli's cells and the interstitial cells appeared normal and no changes were observed in the epididymis.

Skin.—Grossly, beside multiple petechiae, ecchymoses, and subepidermal hemorrhages, there were ulcers of varying sizes with ragged edges and necrotic bases without induration. Loss of hair was sometimes observed in the ulcerated areas, particularly on the extremities.

Microscopically, the usual cellular infil-

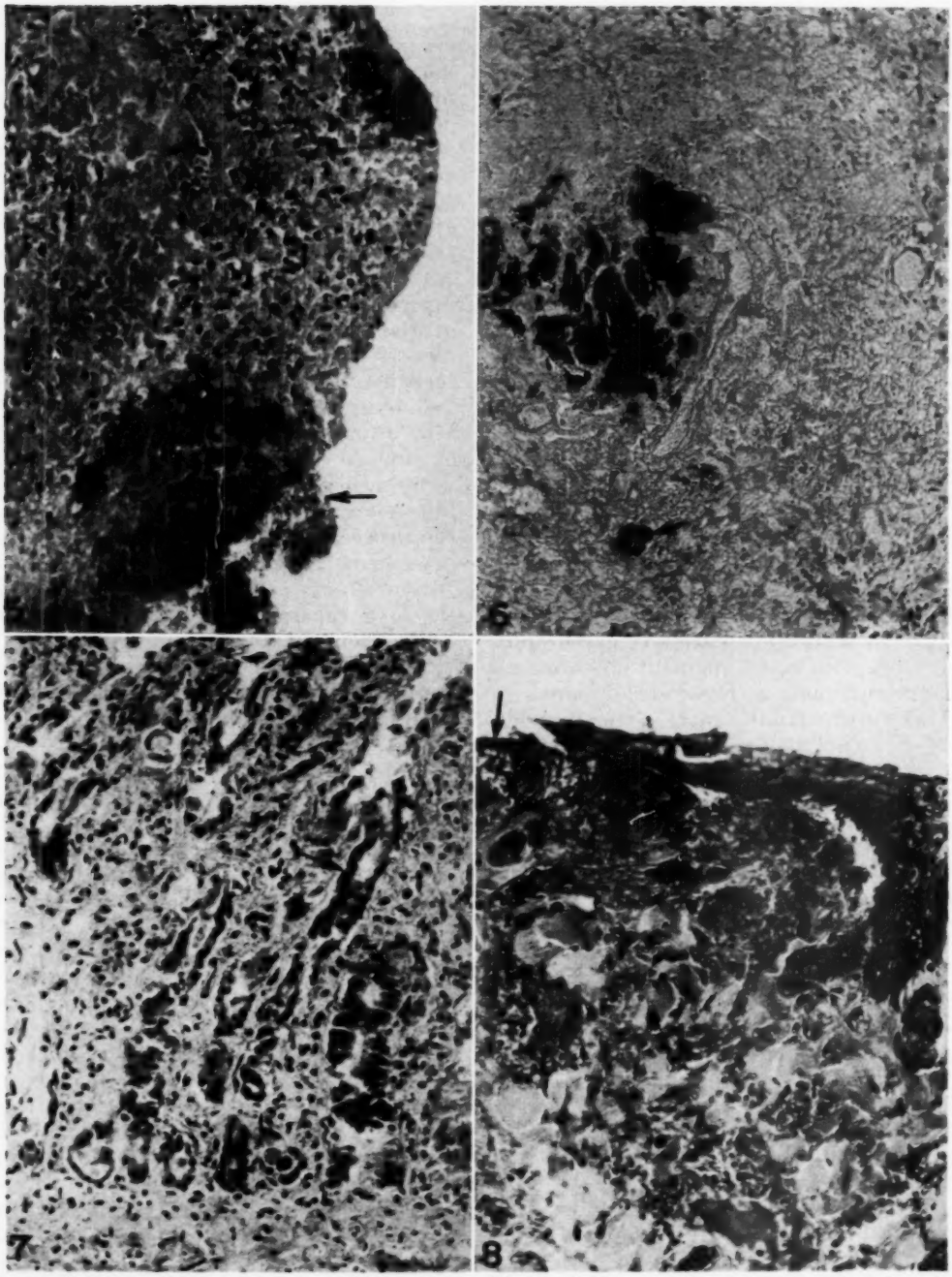


Fig. 5—Section of the tonsil of a dog twelve days after receiving 400 r of total body radiation—ulceration, hemorrhage, necrosis, and bacterial growth with no cellular response. x 186. AFIP 248309.

Fig. 6—Section of the lung of a dog nine days after receiving 600 r of total body radiation—marked bacterial browth in area of necrosis without any cellular response. x 90. AFIP 248123.

Fig. 7—Section of the small intestine of a dog three days after receiving 100 r of total body radiation — degeneration of glandular epithelium. x 186. AFIP 248187.

Fig. 8—Section of the tongue of a dog eighteen days after receiving 400 r of total body radiation — ulceration with no cellular response; thrombosis of vessels. x 186. AFIP 248255.

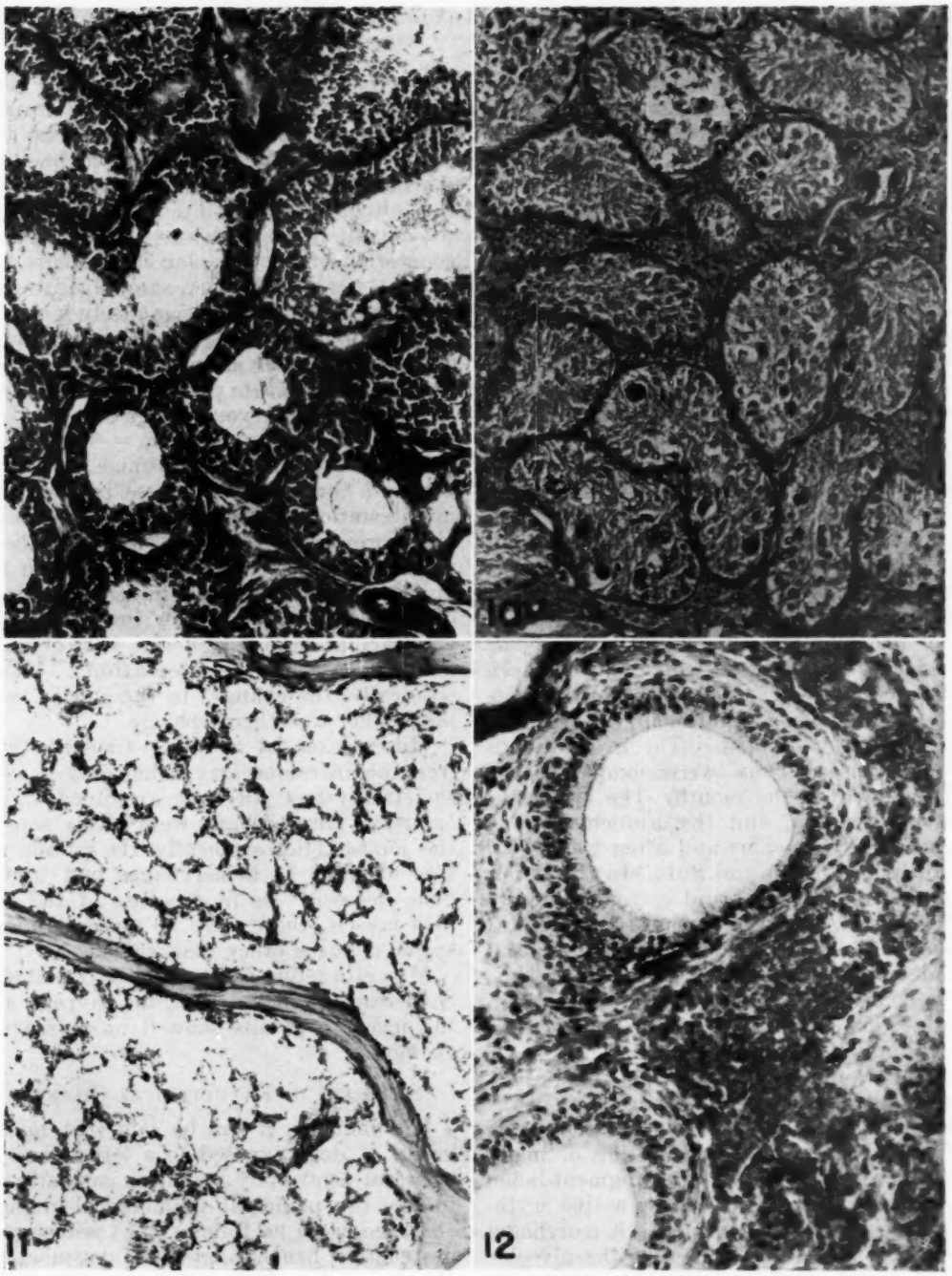


Fig. 9—Section of the testis of a dog eleven days after receiving 600 r of total body radiation—decrease in spermatogenesis and degeneration of germinal epithelium. x 100. AFIP 248127.

Fig. 10—Section of the testis of a dog twelve days after receiving 600 r of total body radiation—focal spotty atrophy of seminiferous tubules, occasional bizarre germinal cell present, interstitial cells and Sertoli's cells persist. x 100. AFIP 248125.

Fig. 11—Section of bone marrow from the rib of a dog nine days after receiving 600 r of total body radiation — aplastic bone marrow. x 90. AFIP 248123.

Fig. 12—Section of the epididymus of a dog eight days after receiving 300 r of total body radiation—interstitial hemorrhage. x 186. AFIP 248216.

tration was completely lacking in the ulcers and, frequently, large colonies of bacteria were seen within the tissues. The petechiae, ecchymoses, and large hemorrhagic areas were similarly lacking in cellular and inflammatory response.

Urinary Tract.—Grossly, the kidneys were often involved by extrarenal hemorrhagic processes: petechiae, ecchymoses, or massive hemorrhages. Peripelvic and periureteral hemorrhages were common. The renal parenchyma showed very little gross change with the exception of congestion, demonstrated by numerous petechiae within the parenchyma. The urinary bladder was often the site of a hemorrhagic process, most often in the submucosa, which in many instances involved all layers of the bladder and the perivesical fat. In several animals, the perivesical and perirectal hemorrhage was so extensive that it is believed to have caused obstruction of both urethra and rectum. Microscopically, no specific changes were observed which had not been observed grossly. Congestion and hemorrage were the principal changes.

Respiratory System.—The gross lesions in the nasopharynx were comparable to those noted in the mouth. The epiglottis, larynx, trachea, and the bronchi showed submucosal petechiae and often were filled with bloody froth and fluid. In the lungs, subpleural petechiae and ecchymoses were prominent. The pulmonary parenchyma was generally edematous with patchy areas of decreased crepitation. There were massive hemorrhages in the lungs of many animals. The bronchial lymph nodes were large and hemorrhagic.

Microscopic examination of the pulmonary parenchyma revealed congestion of the alveolar capillaries and blood vessels, edema of the alveolar walls, and filling of many alveoli with fluid containing pigment-laden macrophages, some exhibiting active erythrophagocytosis. In some areas, hemorrhages involved both the alveoli and the alveolar walls. Colonies of bacteria that had excited no inflammatory cellular infiltration were present. Few areas of necrosis were seen. Many of the terminal bronchioles were plugged with mucus and serosanguineous material, including cellular debris.

Gastrointestinal Adnexa.—The liver and extrahepatic biliary system, including the gall bladder and the pancreas, were grossly normal except for occasional peripancreatic hemorrhage. Microscopically, passive congestion was the only evidence of injury observed in the liver and pancreas. This meager change was also observed in other specialized epithelial structures such as the salivary glands.

Central Nervous System.—Other than congestion of the vascular components, the central nervous system showed little anatomical change. Petechiae rarely were apparent in the meninges. Microscopically, with conventional staining methods, there was little to indicate pathological alerations in the central nervous system.

Cardiovascular System.—The heart showed subepicardial petechiae, ecchymoses, and hemorrhages. These hemorrhagic manifestations seemed to be associated with the coronary circulation for only occasional small hemorrhages were seen in the myocardium. Fibrinosanguineous pericarditis was evident in a few instances. The endocardium and valves were sites of minimal hemorrhagic manifestations. Microscopically, the changes in the cardiovascular system were hemorrhagic.

Musculoskeletal System.—Grossly, there were no intramuscular hemorrhages. The skeletal system was not examined in its entirety. Hemorrhages were rarely seen in the joints. Microscopically, the muscle tissue adjacent to hemorrhages had undergone necrosis. As in the case of the cardiac muscle, there were no striking microscopic changes other than edema.

Miscellaneous Specialized Epithelial Structures.—The adrenal, the thyroid, and the prostate glands showed no significant changes.

SUMMARY OF PATHOLOGICAL CHANGES

In summary, it can be said that in this group of dogs exposed to a lethal dose of bilateral total body radiation and allowed to die, the principal pathological changes observed were as follows: (1) widespread destructive hemorrhagic and degenerative changes within the lymphoid elements, with practically no evidence of attempts at regeneration; (2) widespread degeneration within the hematopoietic elements of the bone marrow with no signs of regeneration; (3) extensive degenerative and hemorrhagic manifestations within the mucosal elements of the gastrointestinal tract, particularly the small intestine and terminal colon, with ulceration and hemorrhage;

(4) generalized late evidence of a hemorrhagic tendency, manifested in the form of petechiae, ecchymoses, and massive hemorrhages; (5) bacterial invasion of the tissues inciting no cellular response; (6) patchy degeneration of testicular parenchyma with hemorrhage and degeneration of the germinal epithelium and suppression of spermatogenesis; (7) relative radioresistance of certain types of epithelial tissue such as pancreas, liver, and salivary, adrenal, prostate, and thyroid glands; (8) apparent radioresistance of the central nervous system tissues generally; (9) gross and microscopic evidence of extrarenal late hemorrhagic disease with limited gross and microscopic changes, principally hemorrhagic, within the renal parenchyma; (10) necrosis and ulceration of certain tissues with no cellular response.

References

[1] Gleiser, C. A.: The Sequence of Pathological Events in Dogs Exposed to a Lethal Dose 100/30 of Total Body X-Radiation. Am. J. Vet. Res., *14*, (1953): 287-297.

[2] Gleiser, C. A.: The Determination of the Lethal Dose 50/30 of Total Body X-Radiation for Dogs. Am. J. Vet. Res., *14*, (1953): 284-286.

Clinical Electrocardiograms of the Dog

O. A. SOAVE, D.V.M.

Berkeley, California

THIS STUDY was made to ascertain to what extent electrocardiogram tracings could be used in practice to supplement clinical diagnoses and if they would be indicative of existing pathological alterations prior to clinical symptomatology.

The objectives have been limited to a determination of the normal electrocardiogram and to an investigation into the deviations from normal caused by an upset in the serum-electrolyte balance as seen in renal disease and uremia.

In the course of this work other information was gathered, such as the influence of age and infectious diseases upon the electrocardiogram of the dog, but at the present time this information is beyond the scope of this presentation.

METHOD

A cardiotron,* direct writing model of electrocardiogram was employed. Three standard and two precordial leads were routinely used.

In the standard leads, the electrodes were attached to the right and left forelimbs at a point on the distal third of the radio-ulnar bones and to the left hind limb on the medial surface of the distal end of the tibial bone. These attachments were paired to form lead I (the forelegs), lead II (right fore- and left hind legs), and lead III (left fore- and left hind legs).

In the precordial leads, the electrode close to the heart was placed in the position over the apex of the heart, this being in the sixth intercostal space at the left margin of the sternum. The exploring electrode was attached to either the left hind leg (lead C6LF**) or to the right foreleg (lead C6LR**) as previously described.

All of the animals were laid on a table with the right side down.

THE NORMAL ELECTROCARDIOGRAM

Tracings were procured on 50 apparently normal, healthy animals to establish the normal electrocardiogram so that deviations from this normal might be recognized and interpreted in clinical cases.

In the normal electrocardiogram (table 1) of the dog,[1] it is not unusual to obtain inverted T† waves in all three of the standard leads and prominent Q and S waves in leads II and III and in the precordial lead C6LF. Standard lead I is usually relatively small as far as the QRS complex is concerned, due probably to the fact that the heart is rather centrally placed in the thorax.

A recent publication from the New York State Medical School,[2] Albany, presents a study of the normal electrocardiogram of 30 dogs. Some slight variation in their information and the data given here exists, but it has been found that durations and amplitudes of the electrocardiogram on a single animal will vary slightly if taken at different times. This is due to various environmental and psychogenic factors such as temperature, position, noise, state of excitability, et cetera.

The normal heart rate found exceeds that given by Dukes.[3] To determine the dog's heart rate, hundreds of dogs were examined, both by palpation and through the use of the electrocardiogram. Few heart rates were found to be under 100 beats per minute. However, lower rates will be registered if an animal is adjusted to its surroundings, if no disturbing factors are present, and if the same animal is checked periodically for heart rate over a period of many weeks.

CLINICAL FINDINGS

Pathological, metabolic, and organic changes which cause an alteration in the electrolyte levels in the serum will frequently result in the presentation of an abnormal electrocardiogram. The ions usually affected by these changes are potassium, sodium, and the chlorides. These ions have as one of their functions the regulation of the heart beat and the heart rate.

The heart is bathed in a solution of these ions[4,5] and they play a part in the transmission of the waves of depolarization and repolarization to the electrodes of the electrocardiogram. Any change in their concentration may manifest itself by a corresponding change being recorded on the tracing.

In nephritis or nephrosis, and especially in uremia, the serum-electrolyte balance is altered, due to abnormal filtration by the glomeruli or impaired function of the

Assistant public health veterinarian, Department of Public Health, State of California, Berkeley.

* Electro-Physical Laboratories, Inc., New York, N. Y.

** C refers to the chest, the 6 to the sixth intercostal space, L to the left side of the chest, F to the left leg, and R to the right leg.

† P, Q, R, S, and T are wave designations on the electrocardiogram.

tubules. The electrocardiogram changes seen are usually produced by a potassemia (hyperkalemia). Potassium retention or a rise of the potassium level in the serum usually occurs along with an upset in the sodium and/or chloride levels in the serum during the course of the nephritis, nephrosis, or uremia.

TABLE 1—Findings from Electrocardiograms Procured from 50 Apparently Normal, Healthy Dogs

Waves	Amplitude (mv.) in lead				
	I	II	III	C6LF	C6LR
P	0.15	0.20	0.11	0.15	0.21
Q	0.14	0.21	0.12	0.16	0.18
R	0.7	1.70	1.00	1.40	1.90
S	0.06	0.09	0.18	0.16	1.00
T	0.05	0.14	0.15	±.12	±.18

Duration of waves (seconds)				
P	P-R	QRS	Q-T	Heart rate
0.04-0.08	0.08-0.10	0.03-0.06	0.16-0.21	80-160

One of the functions of potassium is its association with acetyl choline at the myoneural junction; the presence of potassium is necessary for impulse conduction and muscular contraction of the heart.

In potassemia, the conduction of the beat impulse is depressed so that some degree of bradycardia, or slowing of the heart rate, may be evidenced. The T waves are of longer duration and a resulting increase in the Q-T interval occurs. An increase in the Q-T interval associated with clinical evidence is indicative of electrolyte imbalance with potassium retention. The Q-T interval in well-developed cases of renal damage is usually over 0.22 seconds.

In a study of the electrocardiogram of 25 cases of clinically diagnosed nephritis or uremia, 18 presented prolonged Q-T intervals ranging from 0.22 to 0.28 seconds, and over 75 per cent of these cases showed some degree of bradycardia. Bradycardia is not always present since many of the cases of nephritis seen occur in older animals who have some cardiac involvement or organic disease which tends to produce a tachycardia or increased heart rate.

DISCUSSION AND SUMMARY

Lannek,[6] in his study of electrocardiograms in the dog, found Q-T intervals of over 0.20 seconds. A prolonged Q-T interval *per se* is not indicative of an abnormal condition unless coupled with clinical evidence of disease or unless the Q-T interval is found to be increasing in duration in two electrocardiograms taken at different times.

The Q-T interval is directly proportional to the heart rate. A rapid heart rate produces a shorter Q-T interval and, conversely, a slower heart rate, longer Q-T intervals.

In a recent study correlating serum-potassium levels with electrocardiogram tracings in human beings,[7] 22 of 25 patients who had either renal disease or uremia also had upper normal or high serum-potassium levels. These findings would seem to support the evidence presented here, that the changes seen in the electrocardiogram of the dog in renal disease and in uremia are probably caused by a potassemia (hyperkalemia), and that this increase in the potassium level in the serum usually produces an increase in the Q-T interval, an increase in the height of the T wave, and a peaking of the T waves.

References

[1] Johnston, F. D., Medical School University of Michigan: Personal communication.

[2] Horwitt, S. A., Spanier, M. R., Wiggers, H. C.: The Electrocardiogram of the Normal Dog. Proc. Soc. Exptl. Biol. Med., *84*, (1953): 121.

[3] Dukes, H. H.: The Physiology of Domestic Animals. 3rd ed. Comstock Publishing Co., Inc., Ithaca, 1935.

[4] Best, C. H., Taylor, N. B.: Physiological Basis of Medical Practice. 2nd ed. William Wood and Co., Baltimore.

[5] Birch, G., and Winsor, T.: A Primer of Electrocardiography. Lea and Febiger, Philadelphia, 1946.

[6] Lannek, N.: Clinical and Experimental Study of the Electrocardiogram of the Dog. Royal Veterinary Institute, Stockholm, Sweden, 1949.

[7] Westlake, R. E., Chiu, G. C.: Single Electrocardiogram in the Interpretation of Serum Potassium. New York State J. M., *54*, (1954): 239.

Metopon Analgesia in the Dog

GLENWOOD P. EPLING, D.V.M., M.S., and A. DONALD RANKIN, D.V.M., M.S.

Fort Collins, Colorado

METOPON (methyldihydromorphinone)* is a derivative of morphine which has been known since 1938.[21] It has been used as an analgesic in human medicine on terminal cancer patients for several years but has not been used clinically in animals.

LITERATURE CITED

Although much is written concerning pain, it is difficult to define satisfactorily. Because of this, there is conflict in the literature regarding the reception and perception of pain. Some individuals do not perceive pain at all. Kunkle[17] stated that in man such a condition was usually a malfunction or defect of the central nervous system. Chapman and Jones[5] found a variation of pain reception and reaction in human subjects. They related some of these variations to age, race, and mental states. The Hardy, Wolff, Goodell group[13, 25] showed that neurological, pharmacological, and psychological factors altered the pain threshold. In 1948, Wolff and Wolf[27] defined "pain threshold" as the lowest perceptible intensity of pain, and indicated that the pain threshold stimulus is the energy required to induce threshold pain.

In 1943 Goetzl, Burrill, and Ivy[10] reviewed all the methods for the investigation of pain. Deep and superficial pain can be elicited by electrical, thermal, chemical, and mechanical stimuli. Gordon and Whitteridge[11] have described a method of measuring the conduction time for painful cutaneous stimulation. Disruption of the *alpha* rhythm of the electroencephalogram was used as an indicator of cortical disturbance.

One of the more commonly used means of studying pain threshold was described by Hardy, Wolff, and Goodell in 1940.[14] This utilized a light bulb as the source of a potentially variable thermal stimulus to which the skin of the subject was exposed for varied periods of time. Modifications of this method have been used by Ercoli and Lewis[9] on rats, by Andrews and Workman[3] on dogs, and by D'Amour and Smith.[6] Andrews and Workman stated that pain threshold studies in dogs gave results comparable to those in human subjects and that drugs affected the thresholds much as in man.

Evidence available from studies with various devices indicates that under experimental conditions healthy persons all have approximately the same pain perceiving capacity; that the pain threshold in man is independent of sex; and that the pain threshold is uniform throughout the day or even the year.[15]

Metopon is chemically known as methyldihydromorphinone and is most commonly supplied in the form of a hydrochloride. It is a synthetic derivative of morphine. The human dose of 7.0 mg. was first reported by Small, Eddy, Mosettig, and Himmelsbach in 1938.[21] These authors reported an absence of emetic action and no adverse respiratory effect at that dose.

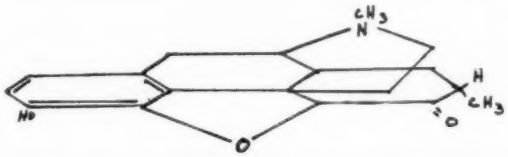


Chart 1—Structural formula of metopon.

In 1939, Himmelsbach[15] reported some addiction characteristics of metopon. His work showed that morphine addiction was not satisfied by metopon substitution. The addiction symptoms for metopon in the dog, as recorded by Eddy,[7] in 1941, were twitching, tremors, muscular weakness, rigidity of voluntary muscles, restlessness, sleepiness, and vomiting. Sumwalt,[23] in 1943, reported doses of metopon used for mice, rats, and cats. The dose given for the cat was based on the minimal amount required to induce excitation and was 0.1 mg. per kilogram of body weight. Beckmann,[3] in 1942, observed that metopon produced less sedation and mental confusion than did morphine, it did not depress respiration to the degree that morphine did, and with its use nausea was infrequent. He also pointed out that metopon was more expensive than morphine, and the effect of a single dose usually lasted only about two hours. In contrast to this, Grollman and Slaughter[12] observed that only half the dose of metopon was necessary to produce the same effect

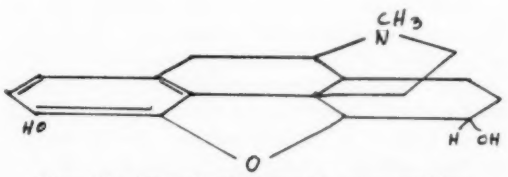


Chart 2—Structural formula of morphine.

From the Departments of Anatomy and Physiology, Colorado A. and M. College, Fort Collins.

The data herein are taken from a thesis submitted by Dr. Epling to the Graduate School, Colorado A. and M. College, Fort Collins, in partial fulfillment of the requirements for the degree of master of science in physiology. August, 1953, and are published with the approval of the dean of the Graduate School.

* Metopon was supplied for this work by the New York Quinine and Chemical Works.

as a full dose of morphine. Sollman,[22] in 1948, credited Small and Mosettig with the development of metopon and stated that its nauseant action was less, its toxicity greater, the analgesia more potent than with morphine, and that the analgesia was distinctly more persistent. Also, in 1947, Bastedo [2] noted that metopon was more analgesic and less addictive than dilaudid.

Small [20] reported the structural formula of metopon, in 1948, as shown in chart 1.

For the purpose of comparison the structural formula of morphine is shown in chart 2.

Eddy, in 1948,[8] stated that, compared to morphine, metopon exhibited an exaggerated analgesic effectiveness and a diminution of sedation, euphoria, emesis, and intestinal activity. He substantiated the infrequency of nausea accompanying metopon medication and indicated that tolerance was slower to develop to metopon than to morphine. Howard, in 1952,[16] stressed the fact that oral administration of metopon was extremely effective and obviated the need for hypodermic injections. Salter, in 1952,[18] substantiated this idea and added that the drug was less somnifacient than morphine.

METHODS AND MATERIALS

In this evaluation of metopon as an analgesic for dogs, 100 dogs of various sizes, ages, and breeds were used. Except for those used in tolerance tests, each animal was used only once and received no other drug prior to the test. Those used in the tolerance tests were injected daily as described later. The drugs used were morphine sulfate and metopon hydrochloride which were injected subcutaneously, observing sterile precautions.

The normal pain threshold was determined for each animal before any drug administration and at intervals following the injection of the analgesic. The method used, essentially that of Hardy, Wolff, and Goodell,[14] was as follows.

The light (and heat) from a 1,000-watt lamp was focused by condensing lenses through a fixed aperture onto the shaved, blackened, lateral surface of the thigh of the dog. The thigh was thoroughly blackened with black liquid shoe polish to insure a uniform absorption of the radiated heat. Between the lens and the aperture a manually controlled shutter was mounted to regulate the length of the exposure (fig. 1). During the testing the subject was held, with minimal restraint, so the blackened thigh was adjacent to the aperture. The shutter was removed and the radiation allowed to fall on the skin until the animal reacted by quick movements in an attempt to escape the painful stimulus. The time interval between the beginning of the exposure and the response was measured with a stop watch and recorded as the pain threshold time.

The time required to raise a blister was determined, and thereafter the animals were subjected to 0.1 to 0.2 seconds less exposure than the time required to blister. Blistering time was found to be constant among animals when using the same equipment but was found to vary from 14.0 seconds with one lamp (with 85 volts input) to 7.1 seconds with another lamp (with 60 volts input). The voltage input was held constant with a variable transformer. Two animals were encountered which did not react in any manner to the stimulus even though blistering occurred. They were rejected from this study.

Following the initial measurement of the pain threshold, rectal temperature, pulse rate, and respiration rate, the dog was injected with the analgesic. In those animals used to determine the optimal analgesic dose of metopon, the pain threshold was determined every ten minutes for three hours. The temperature, pulse rate, and respiration rate were recorded ten minutes after the injection and every thirty minutes thereafter for three hours. The series was started with a dose of 0.1 mg. of metopon per kilogram of body weight

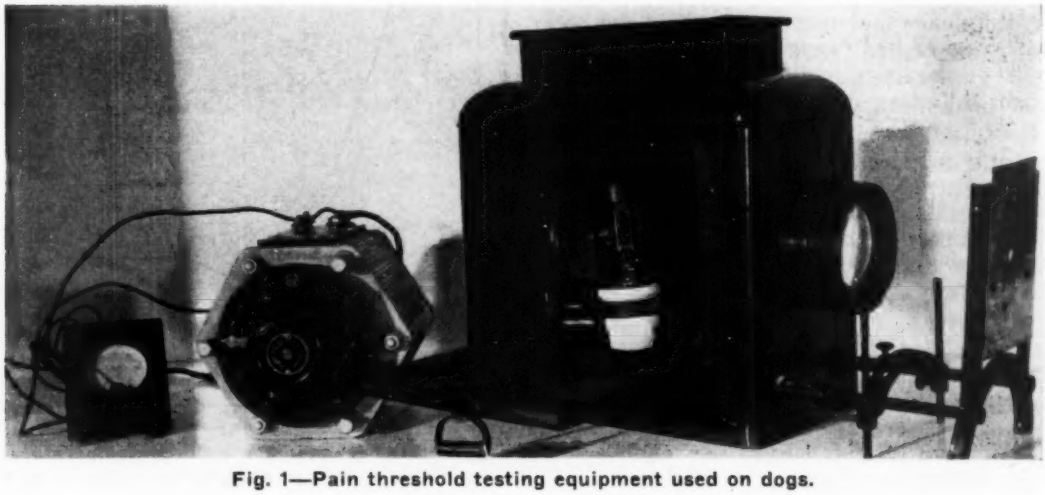


Fig. 1—Pain threshold testing equipment used on dogs.

(the recommended human dose). Because the morphine dose for the dog is much larger than that for man, it was surmised that the same might be true for metopon. Hence, the dose was increased until the optimal dose was found. An optimal do .

TABLE 1—Analgesic Effects of Various Doses of Metopon in the Dog

Dose of metopon (mg./kg. body wt.)	No. dogs receiving dose	No. dogs show. complete analgesia three hours after inject.
2	10	4
3	10	7
4	10	10
5	10	9

was arbitrarily defined as one which would produce complete analgesia for three hours after its administration. Following the establishment of this optimal dose, numerous experiments were performed for verification of the dose.

Similar experiments were performed using morphine as the analgesic so that the two drugs could be compared. The commonly accepted dose of morphine (4 mg. per kilogram of body weight) was used.

To determine whether a tolerance to metopon developed, a series of ten experiments was performed using the same apparatus as previously described. The subjects were given the optimal dose of metopon daily for ten days. During these tests the pain threshold, rectal temperature, pulse, and respiration rates were measured every thirty minutes throughout the duration of complete analgesia of each day's experiment. For this study, complete analgesia was defined as insensitivity to the stimulus for the full duration of exposure, *i.e.*, just short of blistering time.

EXPERIMENTAL RESULTS

The optimal analgesic dose of metopon in the dog was determined to be 4 mg. per kilogram of body weight (table 1).

The average duration of complete analgesia following optimal metopon dosage in the dogs used in this portion of the experiments was 5.6 hours (table 2).

TABLE 2—Duration of Metopon Analgesia in the Dog at Optimal Dose

Dog (No.)	Duration complete analgesia (hr.)
65	7.0
66	5.5
67	7.0
68	7.0
69	3.5
70	7.0
71	5.0
72	4.5
73	5.0
74	4.0
Average	5.6

In determining if a tolerance to metopon was established by repeated daily doses, one factor was considered: the duration of analgesia on ten successive days of optimal dosage. Using this guide, all 10 animals tested developed a tolerance, *i.e.*, each responded to pain more promptly following the injection of metopon after ten days of repeated injections. The average duration of complete analgesia decreased from 5.6 hours on the first day to 1.9 hours on the tenth day. In addition, 8 of the 10 animals used showed a definite weight loss during the ten-day period. One animal remained constant in weight, and 1 showed a slight gain in weight.

A comparison of the action of metopon with that of morphine was made, and the following pertinent differences were noted.

1) Nine out of 10 dogs injected with optimal doses of metopon showed complete analgesia ten minutes following the injection. Only four of the 10 animals injected with optimal doses of morphine showed complete analgesia ten minutes following injection.

2) All 10 dogs injected with optimal doses of metopon showed complete anal-

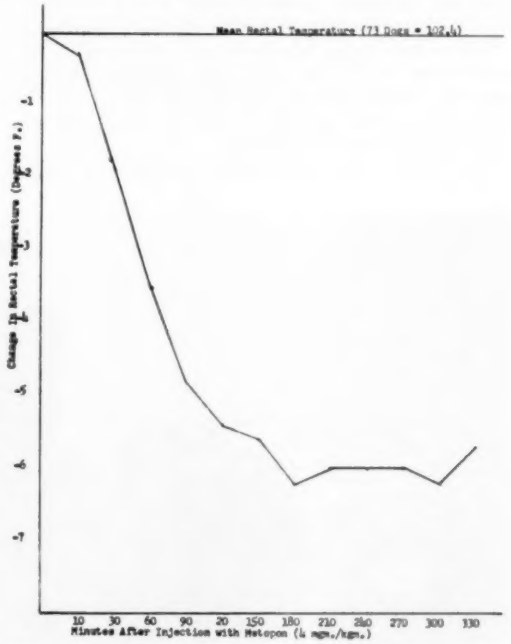

Mean Rectal Temperature (71 Dogs = 102.4)
-1
-5
-6
-7
10 30 60 90 20 150 180 210 240 270 300 330
Minutes After Injection with Metopon (4 mgs./kgm.)

Fig. 2—Effect of metopon on body temperature of dogs.

gesia at the end of three hours, whereas only 5 of the 10 dogs injected with optimal doses of morphine showed complete analgesia at the end of three hours.

These results indicate that the analgesia produced by metopon was more prompt and more persistent than analgesia produced by morphine.

The subcutaneous administration of optimal doses of metopon caused the temperature of the dogs to drop markedly (fig. 2). In the 10 animals used for tolerance testing, the temperatures dropped an average of 6.2 degrees in three hours.

The pulse rate was slowed by the subcutaneous administration of optimal doses of metopon. In the 10 dogs used for tolerance testing, the pulse rate showed a marked drop followed by a slight rise and leveling off (fig. 3). The respiration rates rose abruptly, then fell below normal and leveled off (fig. 4).

Although no records were kept on emesis and defecation following the administration of metopon, it was estimated that about one third of the animals demonstrated either one or both. These might be considered advantageous effects in a drug used for preanesthetic medication, but they could be handicaps in postoperative analgesic performance.

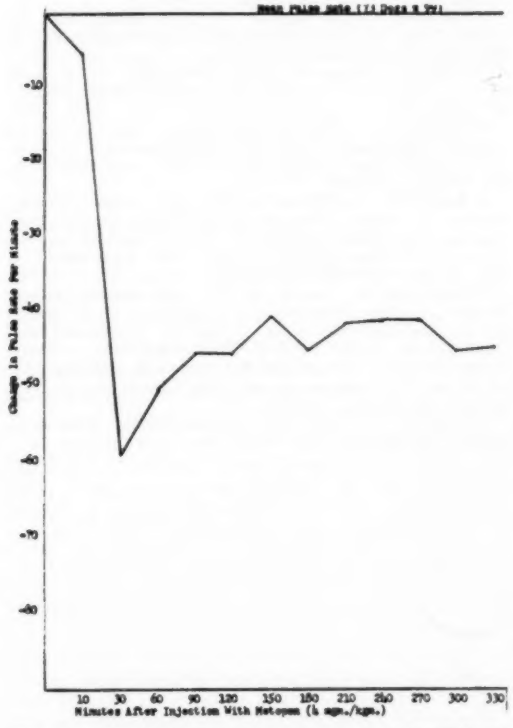


Fig. 3—Effect of metopon on pulse rate of dogs.

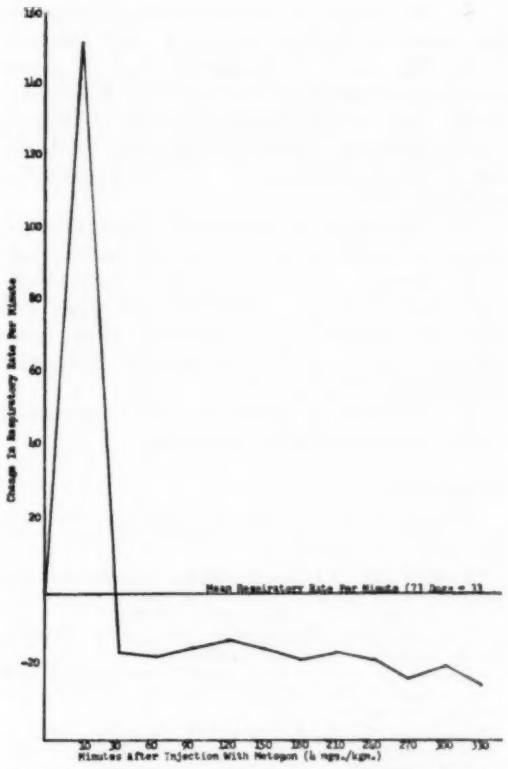

Change In Respiratory Rate Per Minute
Mean Respiratory Rate Per Minute (71 Dogs = 11)
10 30 60 90 120 150 180 210 240 270 300 330
Minutes After Injection With Metopon (1 mgm./kgm.)

Fig. 4—Effect of metopon on respiration of dogs.

Discussion

A question of efficiency of the method of determination of the pain threshold arose in the pursuit of this work. Although an animal might prove unresponsive to thermal stimuli, yet it could react to pinching or tickling. Hence, the ability of the apparatus to measure analgesic drug action in dogs might be disputed. Beecher[4] questioned the use of animals in this type of work. He indicated that the connection between pain-relieving power and depression of various reflexes was not clear. However, it is not practical to use man as a subject to determine the usefulness of drugs on dogs; rather, the reverse is the usual process.

The high cost of metopon and its relative unavailability make it somewhat im-

practical for use in veterinary practice at this time. However, it is possible that demand could alter these situations.

SUMMARY

1) The value of metopon as an analgesic for dogs is studied, utilizing 100 animals.

2) The optimal analgesic dose of metopon in the dog, as determined in this study, is 4.0 mg. per kilogram of body weight.

3) Metopon analgesia persisted in these dogs for an average of 5.6 hours with the optimal dose.

4) Tolerance to repeated injections of metopon seems to occur rapidly in the dog.

5) Results obtained in this study indicate that metopon analgesia for dogs has two advantages as compared with morphine: (1) rapidity of action, and (2) persistence of analgesia.

6) Metopon appears to have potential value as an analgesic for dogs.

References

[1] Andrews, H. L., and Workman, W.: Pain Threshold Measurement in the Dog. J. Pharmacol. and Exptl. Therap., *73*, (1941): 99-103.

[2] Bastedo, W. A.: Pharmacology Therapeutics and Prescription Writing for Students and Practitioners. W. B. Saunders Co., Philadelphia, 1947.

[3] Beckman, H.: Pharmacology in Clinical Practice. W. B. Saunders Co., Philadelphia, 1942.

[4] Beecher, Henry K.: Experimental Pharmacology and Measurement of the Subjective Response. Science, *116*, (1952): 157-162.

[5] Chapman, W. P., and Jones, C. M.: Variations in Cutaneous and Visceral Pain Sensitivity. J. Clin. Invest. *23*, (1944): 81-91.

[6] D'Amour, F. E., and Smith, D. L.: A Method for Determining Loss of Pain Sensation. J. Pharmacol. and Exptl. Therap. *72*, (1941): 74-79.

[7] Eddy, Nathan B.: Morphine: Tolerance and Addiction. U. S. Pub. Health Serv. Pub. Health Rep., Suppl. 165, (1941): 687-758.

[8] Eddy, Nathan B.: Pharmacology of Metopon and Other New Analgesic Opium Derivatives. Ann., New York Acad. Sci., *51*, (1948): 51-58.

[9] Ercoli, N., and Lewis, M. N.: Studies on Analgesics. I. The Time Action Curves of Morphine, Codeine, Dilaudid, and Demerol by Various Methods of Administration. II. Analgesic Activity of Acetylsalicyclic Acid and Aminopyrine. J. Pharmacol. and Exptl. Therap., *84*, (1945): 301-317.

[10] Goetzl, Franz R., Burrill, Daniel Y., and Ivy, A. C.: A Critical Analysis of Algesimetric Methods with Suggestions for a Useful Procedure. Northwestern Univ. M. School Quart. Bull., *17*, (1943): 280-291.

[11] Gordon, G., and Whitteridge, D.: Conduction-Time for Human Pain Sensation. Lancet, *2*, (1953):700-701.

[12] Grollman, A., and Slaughter, D.: Pharmacology and Therapeutics. Lea and Febiger, Philadelphia, 1947.

[13] Hardy, J. D., Wolff, H. G., and Goodell, Helen: Pain Threshold in Man. In "Medical Physics" ed. by Otto Glasser. The Year Book Publishers, Chicago (1947): 901-905.

[14] Hardy, J. D., Wolff, H. G., and Goodell, Helen: Studies on Pain. A New Method for Measuring Pain Threshold: Observations on Spatial Summation of Pain. J. Clin. Invest., *19*, (1940): 649-657.

[15] Himmelsbach, C. K.: Studies of Certain Addiction Characteristics of *(a)* Dihydromorphine (Paramorphan), *(b)* Dehydrosoxymorphine D (Desomorphine), *(c)* Dehydrodesoxycodeine D (Desocodeine), and *(d)* Methyldihydromorphinone (Metopon). J. Pharmacol. and Exptl. Therap., *67*, (1939): 239-249.

[16] Howard, M. E.: Modern Drug Encyclopedia and Therapeutic Index. Drug Publications, New York, 1952.

[17] Kunkle, E. Charles, and Chapman, W. P.: Insensitivity to Pain in Man. Proc. A. for Res. on Nerv. and Ment Dis., *23*, (1943): 100-109.

[18] Salter, W. T.: A Textbook of Pharmacology. W. B. Sounders Co., Philadelphia, 1952.

[19] Schumacher, G. A., Goodell, H., Hardy, J. D., and Wolff, H. G.: Uniformity of Pain Threshold in Man. Science, *92*, (1940): 110-112.

[20] Small, Lyndon F.: Chemistry of Natural and Synthetic Analgesics. Ann. New York Acad. Sci., *51*, (1948): 12-20.

[21] Small, Lyndon F., Eddy, Nathan B., Mosettig, Erich, and Himmelsbach, C. K.: Studies on Drug Addiction, with Special Reference to Chemical Structure of Opium Derivatives and Allied Synthetic Substances and Their Physiological Action. U. S. Pub. Health Serv. Pub. Health Rep. Suppl. 138, (1938): 1-143.

[22] Sollman, T.: A Manual of Pharmacology and Its Applications to Therapeutics and Toxicology. W. B. Saunders Co., Philadelphia, 1948.

[23] Sumwalt, Margaret: Other Alkaloids. U. S. Pub. Health Serv. Pub. Health Rep. Suppl. 165, (1943): 813-1088.

[24] Wolff, H. G., and Goodell, Helen: The Relation of Attitude and Suggestion to the Perception of and Reaction to Pain. Proc. A. for Res. on Nerv. and Ment. Dis., *23*, (1943): 434-448.

[25] Wolff, H. G., Hardy, J. D., and Goodell, Helen: Studies on Pain: Measurement of Effect of Morphine, Codeine, and Other Opiates on Pain Threshold and on Analysis of Their Relation to the Pain Experience. J. Clin. Invest., *19*, (1940): 659-680.

[26] Wolff, H. G., Hardy, J. D., and Goodell, Helen: Studies on Pain: Measurement of Effect on Pain Threshold of Acetylaslicylic Acid, Acetanilid, Acetophenetidin, Aminopyrine, Ethyl Alcohol, Trichlorethylene, a Barbiturate Quinine, Ergotamine Tartrate, and Caffeine: Analysis of Their Relation to the Pain Experience. J. Clin. Invest., *20*, (1941): 63-80.

[27] Wolff, H. G., and Wolf, Stewart: Pain. Charles C. Thomas, Springfield, Ill., 1948.

Electrophoretic Study of the Horse Serum of Equine Infectious Anemia

KUNG YING TANG KAO, M.D., Ph.D.; REGINALD L. REAGAN, Major (Ret.), U.S.A.; A. L. BRUECKNER, V.M.D.

College Park, Maryland

ACCORDING to Gilman,[1] chemical fractionation of the blood serum of horses affected with equine infectious anemia showed an extremely low albumin-globulin ratio (A/G ratio). Hirtz[2] reported that electrophoretic analysis of serum of equine infectious anemia was of no diagnostic value. The difference in their results aroused interest in an electrophoretic study on the serum of equine infectious anemia.

MATERIALS AND METHODS

Serums were supplied by Dr. T. O. Roby of the Pathological Division, Bureau of Animal Industry, Agricultural Research Administration, Washington, D. C. Horse 1529 was inoculated, Oct. 1, 1953, with lyophilized serum containing a Wyoming strain of the virus, reconstituted with 10 ml. sterile distilled water. This lyophilized serum was collected about four years previously from horse 1042, in which acute equine infectious anemia had been produced experimentally by inoculation with a strain of virus initially isolated from a naturally occurring case in Wyoming. The serum of horse 1042 had been filtered through a Mandler No. 8 candle, tested for bacterial sterility by culturing on aerobic and anaerobic mediums, and then lyophilized. Horse 1529 had a temperature of 105.4 F. on Oct. 16, 1953. Blood was taken that same day and a necropsy was performed.

Horse 1531 was inoculated, Oct. 1, 1953, with reconstituted lyophilized serum containing a New Hampshire strain of the virus. This lyophilized serum was collected from horse 1041 in which equine infectious anemia also had been produced experimentally by inoculation on Aug. 23, 1947, with a strain of virus initially isolated from a naturally occurring case at Rockingham Park, N. H. The serum of horse 1041 was filtered through a Mandler No. 6 candle and lyophilized. Horse 1531 had a temperature of 106.2 F. on Oct. 20, 1953. Serum was collected on Oct. 20 and again on Nov. 4, 1953. The horse is still under observation.

On July 16, 1953, horse 1511, which represents a chronic case of equine infectious anemia, was inoculated with reconstituted lyophilized serum containing the Wyoming strain of the virus from horse 1042. An initial febrile reaction occurred on July 27, 1953, and reached a maximum of 106.0 F. in four days and then returned to normal. A second short febrile reaction occurred on Oct. 1, 1953, reaching 104.8 F. before returning to normal. The serum was collected on Nov. 4, 1953. Serums of the 3 diseased horses were passed through Mandler No. 8 candles and were kept at 6 C. Horses 1528, 1532, 1533, and 105 were normal. Their serums were taken aseptically and kept at 6 C. for use as controls.

Electrophoretic Study.—The serums were diluted with equal amounts of veronal buffer of pH 8.6 with an ionic strength of 0.1 and dialyzed against the same buffer at room temperature for two hours.[3] Each sample was centrifuged at 4,000 r.p.m. for ten minutes in a Sorvall angle centrifuge. The clear supernatant fluid was placed in the cell of an Aminco-Stern electrophoresis apparatus at a constant temperature of 2 C., and a 12 milliampere current at 115 volts was passed through it for two hours. The potential gradient used was 4.4 volts/cm. The heat dissipation was 10 watts. Both starting peaks and final patterns were photographed and the negatives were enlarged. The relative percentage of each component was calculated with a KE planimeter by measuring the component area under the corresponding peak of the enlarged picture x 100/total area exclusive of the area under residue peak δ.

Chemical fractionation of the serum protein was also made for comparison with the electrophoretic results. The globulin was precipitated while the preparation was being brought up to a concentration of 26 per cent Na_2SO_4. The procedure is similar to the method described by Hawk.[4]

TABLE 1—Relative Percentages of Components of Serums from Normal Horses and from Horses with Equine Infectious Anemia

Horse (No.)	Serum components: Albumin (%)	Globulin (%) α_1	α_2	β	γ	A/G* ratio
NORMAL HORSES						
1528	38.71	19.81	19.36	12.00	10.12	0.63
1532	41.15	16.05	11.05	14.40	17.35	0.74
1533	37.83	15.43	14.08	16.23	16.43	0.61
105	40.62	16.37	15.42	11.72	15.87	0.69
HORSES WITH EQUINE INFECTIOUS ANEMIA						
1529	36.43	17.23	9.68	12.63	24.03	0.57
1531**	32.59	21.74	9.89	13.29	22.49	0.49
1531†	30.87	18.87	11.42	12.47	26.37	0.45
1511	23.78	17.48	11.88	10.78	36.08	0.31

* Albumin-globulin ratio. ** Oct. 20, 1953. † Nov. 4, 1953.

From the Virus Laboratory, Live Stock Sanitary Service, University of Maryland, College Park.

Nitrogen determination of total protein and of albumin was done by the Kjeldahl's method.[4]

RESULTS AND DISCUSSION

The electrophoretic patterns are shown in figure 1. The mobilities of each component of horse serum in cm.^{2}/sec./volt x 10^{-5} are: albumin 9.32, α_1-globulin 6.22, α_2-globulin 5.15, β-globulin 3.88, γ-globulin 1.92. Patterns of serum from horses (1529 and 1531) with acute equine infectious anemia show a relatively elevated γ-globulin peak. A very low albumin and a very high γ-globulin peak are shown in the pattern of serum from the chronic equine infectious anemia of horse 1511. These patterns of serum from horses with equine infectious anemia show that as the anemia proceeds the relative amount of albumin decreases and the relative amount of γ-globulin increases. The planimetric results are shown in table 1. From the acute cases of equine infectious anemia in horses 1529 and 1531, relatively low albumin, relatively low α_2-globulin, and relatively high γ-globulin contents are shown with the corresponding decrease in the A/G ratio. A very low albumin, a very high γ-globulin, and very low A/G ratio are shown in the chronic case of equine infectious anemia of horse 1511.

The chemical fractionation was done for comparison with the planimetry. In table 2, the A/G ratios are relatively low in the acute cases and extremely low in the

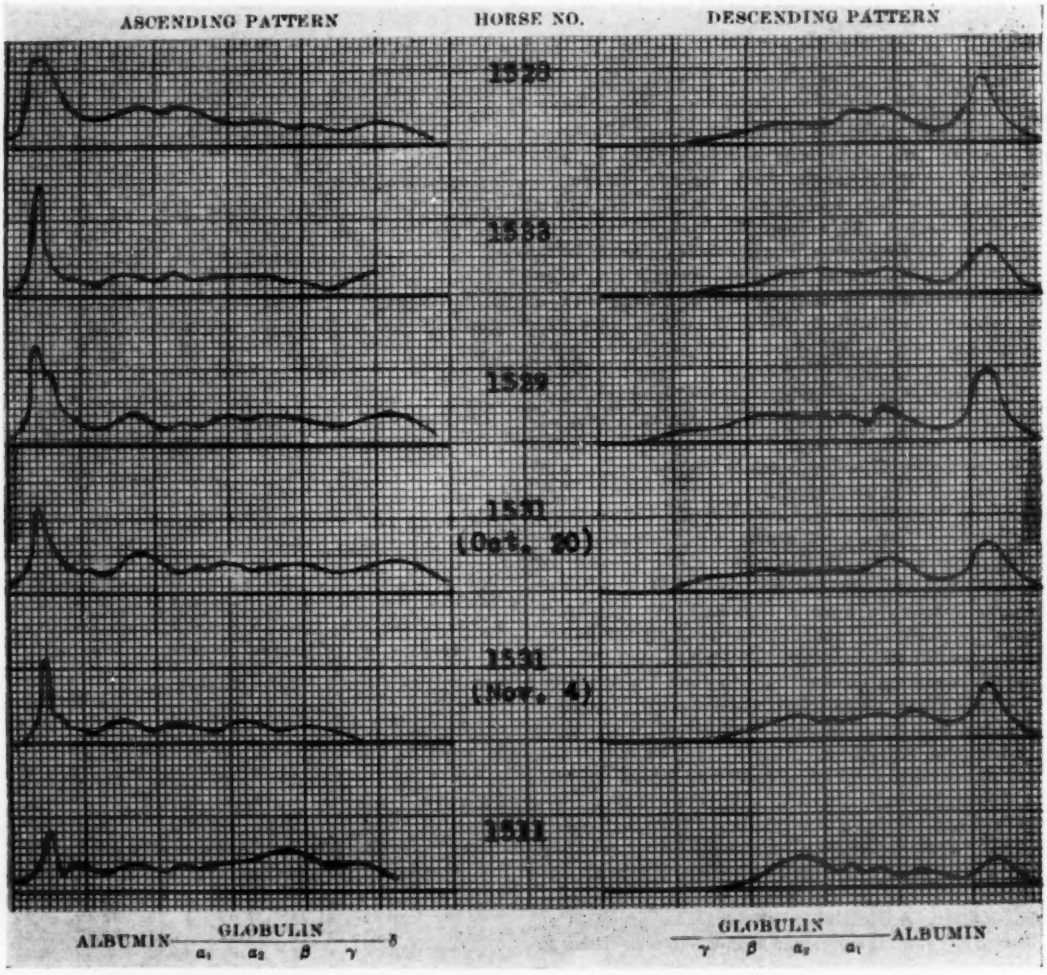


Fig. 1—Electrophoretic patterns of normal and infectious anemia horse serums.

chronic case of equine infectious anemia. The results are not completely parallel to the planimetry.

Jager *et al.*[5] have reported that the electrophoretic pattern of the albumin fraction obtained by the 26 per cent Na_2SO_4 precipitation method consists not only of albumin but also some α_1-, α_2-, and β-globulin. No study has been made on the electrophoretic pattern of the globulin fraction obtained by the 26 per cent Na_2SO_4 precipitation method. The discrepancy of the results in the present study may be due to the impure mixtures obtained by the chemical method of fractionation. The electrophoretic separation of serum protein is by far the best method to evaluate the composition of serum protein.

TABLE 2—Composition of Serum Proteins and Albumin-Globulin Ratio of Normal Horses and of Horses with Equine Infectious Anemia Determined by the Chemical Method

Horse (No.)	Total serum protein (Gm./100 ml.)	Albumin (Gm./100 ml.)	Globulin (total protein-albumin) (Gm./100 ml.)	A/G* ratio
NORMAL HORSES				
1528	6.47	2.81	3.66	0.77
1532	6.33	3.08	3.25	0.95
1533	7.37	2.79	4.58	0.61
HORSES WITH EQUINE INFECTIOUS ANEMIA				
1529	7.37	3.00	4.37	0.69
1531**	7.07	2.71	4.36	0.62
1531†	6.85	1.99	4.86	0.41
1511	7.15	1.15	6.00	0.19

* Albumin-globulin ratio. ** Oct. 20, 1953. † Nov. 4, 1953.

SUMMARY

1) The albumin-globulin ratio (A/G ratio) decreases progressively in equine infectious anemia serum as the disease proceeds to the chronic form. This confirms Gilman's study by the chemical method.[1]

2) The experiment shows a decrease of the A/G ratio which is caused by a relative increase of γ-globulin as in the acute case of equine infectious anemia, and a definite increase of γ-globulin and a decrease of albumin as in the chronic form of equine infectious anemia.

3) The A/G ratio calculated from the 26 per cent Na_2SO_4 precipitation method does not parallel that calculated from the electrophoretic pattern.

References

[1] Gilman, A. R.: Plasma Protein Changes in Equine Infectious Anemia. Am. J. Vet. Res., *8*, (1952): 83.

[2] Hirtz, J.: Étudé electrophorétique du sérum de chevaux infectés experimentement d'anémie infectieuses. Rev. Immunol., *16*, (1952): 397-405.

[3] Reiner, M., and Fenichel, R. L.: Dialysis of Protein Solution for Electrophoresis. Science, *108*, (1948): 164-166.

[4] Hawk, P. B., Oser, B. L., and Summerson, W. H.: Practical Physiological Chemistry. 12th ed. The Blakiston Co., Philadelphia, (1947): 546-549, 820-822.

[5] Jager, B. V., Schwartz, T. B., Smith, E. L., Nickerson, M., and Brown, D. M.: Comparative Electrophoretical and Chemical Estimations of Human Serum Albumin. An Evaluation of Six Methods. J. Lab. and Clin. Med., *35*, (1950): 76-86.

Research on Foot-and-Mouth Disease. VII. Cultivation of the Virus on a Practical Scale in Explantations of Tongue Epithelium

A Modification of the Culture Technique

H. S. FRENKEL

Amsterdam, Holland

IN THE CULTIVATION of virus in tissue cultures or in explantations of tissue kept alive *in vitro*, it has been a common procedure to mince this tissue very finely. As can easily be understood, the mincing of tissue in the cultivation of virus was adopted because the same procedure was used in the technique of tissue cultivation and, over the years, many investigators have been employing this method.

In 1936, Frenkel and van Waveren [1] in their research on the cultivation of the virus of foot-and-mouth disease observed several times that it could be propagated in rather large pieces of embryonic skin of bovine or ovine origin and that eventually mincing could be omitted, the explantations supporting viral growth. As, at that time, their facilities for titration of the virus were insufficient and the complement-fixation test for foot-and-mouth disease virus had not yet been developed to a point that it could be used for titrations, they were unable to compare properly the titer of virus cultivated in minced and unminced tissue. Recently this study was renewed and the results are given in this paper.

MATERIALS AND METHODS

It was shown, first tentatively on a small number of cultures by previously reported techniques [2-4] and later on a large scale by a more recent procedure,[5] in which a 600-liter vaccine-production autoclave was used, that a culture medium containing unminced sheets of mucous membrane from bovine tongues yielded a virus with a titer at least as great but usually greater than the virus produced in a culture medium containing minced tissue. The titrations were made in cattle by Henderson's method.[6] Examples of comparative titration results are shown in table 1.

When using tissue sheets instead of finely minced epithelium, the system of stirring by rotation could no longer be employed. It was obvious that rotation would result in winding of the tissue sheets around the axis, which would make respiration and survival of cells impossible. To meet the aeration requirements, it was necessary to change the stirring system. Instead of stirring by rotation, a vertical stirring was used. This was effected by attaching a motor with an eccentric device on the lid of the culture tank, to which an axle was fixed carrying at the lowest point a stainless steel disk with a diameter 50 mm. less than the inside diameter of the container. By this construction, the disk was moved up and down but it never came above the surface of the nutrient liquid. When the disk reached its lowest point, the tissue sheets appeared in the circular space outside the disk and, when the disk reached its highest point, the tissue disappeared under it. With this procedure, a very good stirring of the tissue was obtained and clumping was avoided. A stainless steel tube with a small number of fine holes fitted in the bottom of the tank was used for aeration.

After the incubation period of about twenty hours, the tissue with the liquid was removed. This may be accomplished either through a tube, at the lowest point of the bottom, wide enough to permit the rather bulky material to pass or by removing the tissue from the container through an opening in the top. As we used an old 600-liter vaccine-production autoclave as a culture tank, we were obliged to follow the latter procedure which is more time consuming.

In order to facilitate the cleaning of the tank which was done in our case without removing the lid, the disk was constructed so that it could be given a perpendicular position.

TABLE 1 — Comparative Titers of Foot-and-Mouth Disease Virus Propagated in Series in Mediums Prepared with Unminced and Minced Bovine Tongue Epithelium

Virus using unminced epithelium		Virus using minced epithelium	
Type and identification No.	Titer *	Type and identification No.	Titer *
A 247	1:1,170,000	A 246	1:510,000
O 105	1:440,000	O 106	1:260,000
C 81	1:200,000	C 82	1:158,000
O 109	1:740,000	O 110	1:460,000

* Titrations made by Henderson's Method [6] (50 % end point in cattle).

From the State Veterinary Research Institute, Amsterdam, Holland. The author is director of the Institute.

Doubtless, the culture tanks with this perpendicular stirring system may also be used for vaccine preparation, which would lower the cost of production materially.

Results and Discussion

The opinion that mincing of tissue produces a large surface and, consequently, a better opportunity for the virus to penetrate the cells seems to be erroneous. On the contrary, by mincing, innumerable small wounds are produced and cells thus destroyed are unable to contribute to virus multiplication. If the sheets of tissue are sufficiently thin, the virus may easily penetrate the cells. The advantages of nonclipping are as follows:

1) More virus is produced.

2) Much unnecessary work is eliminated. The clipping with six electrically driven scissors of about 300 mucous membranes takes about six hours and may provide a greater opportunity of airborne infection with microorganisms.

Thus far, in our laboratory, the sheets of mucous membrane cut off with a slicing machine were collected in small glass jars, each containing a total of four mucosae. These jars had to be sterilized previous to their use, partly filled with nutrient medium, and handled during the clipping process.

When the culture was composed, each jar had to be completely filled with nutrient medium and emptied by suction into the cultivation tank.

In order to avoid too much mechanical action on the tissue, the size of the scissors could not be too large and, consequently, only small jars were necessary. Exaggerated mechanical action is also a factor that kills many tissue cells. Observations had been made that strong mechanical action on the tissue to be minced always resulted in a poor virus culture or in no production of virus at all.

3) When it was found that mincing was no longer necessary, consideration was given to the possibility of using large containers instead of the small jars for the collection of the tissue sheets of the tongue epithelium cut by the slicing machine.

For this purpose, use was made of stainless steel, sterilized 40-liter containers that were filled with nutrient liquid to which adequate quantities of penicillin and streptomycin were added. The filled containers were previously cooled in the refrigerator room. There was no appreciable rise in temperature of the medium during the time when the epithelial sheets were cut. In each of these containers, the epithelial tissue of 50 tongues could be collected.

4) The collection of the sliced sheets by hand with forceps was no longer necessary. By cutting a groove in the circular knife of the slicing machine and fitting into this groove a bronze plate, the sheets of tissue fell directly into the container through a relatively small opening in the lid. This groove was 2.3 cm. from the cutting edge and was about 2 mm. in both depth and width.

After having collected enough tissue to compose a culture in the cultivation tank which needed an amount of epithelium of at least 200 tongues (four containers of 40 liters), the containers were emptied into the culture tank through a large sterile stainless steel funnel. Finally, seed virus was added and the tank closed.

For the harvesting of the virus, it was unnecessary to mince the material in a meat cutter since sufficient grinding and expressing of the tissue was effected by the roller machines.

5) Another advantage of this procedure is that epithelial tissue imported from other countries [7] may be used immediately upon receipt for virus cultivation.

Summary

In the mass production of foot-and-mouth disease virus *in vitro* in which epithelial tissue from bovine tongues forms a part of the medium, tests have shown that fine mincing of the epithelial tissue is unnecessary. When long thin sheets of tongue epithelium as obtained with a slicing machine are used, the yield of virus is either as great or greater than when the tissue is finely minced. In addition, the amount of labor required is less and conditions for maintaining sterility are improved.

A description is given of a mechanical device in the form of a disk moved perpendicularly by a motor with an eccentric attached to the lid of the culture vat, whereby stirring of the tissue in a liquid

medium is accomplished by a vertical rather than a rotating movement.

References

[1] Frenkel, H. S., and van Waveren, G. M.: Jaarverslag van het Staats Veeartsenijkundig Onderzoekings Instituut, 1936-1937.

[2] Frenkel, H. S.: Research on Foot-and-Mouth Disease. II. The Cultivation of the Virus in Explantations of Tongue Epithelium of Bovine Animals. Am. J. Vet. Res., *11*, (1950): 371-373.

[3] Frenkel, H. S.: Research on Foot-and-Mouth Disease. III. The Cultivation of the Virus on a Practical Scale in Explantations of Bovine Tongue Epithelium. Am. J. Vet. Res., *12*, (1951): 187-190.

[4] Frenkel, H. S., and Dunne, H. W.: Research on Foot-and-Mouth Disease. Cultivation of the Foot-and-Mouth Disease Virus in Explanted Epithelium of the Bovine Tongue. IV. Tissue and Medium Variations. Am. J. Vet. Res., *13*, (1952): 21-23.

[5] Frenkel, H. S.: Recherches sur la fièvre aphteuse. La culture du virus sur une échelle pratique par explantation d'épithélium lingual bovin. Bull. Off. internat. Epizoot., *39*, (1953): 91-97.

[6] Henderson, W. M.: The Quantitative Study of Foot-and-Mouth Disease Virus. Rep. Agric. Res. Council (Ser. No. 8), London, 1949.

[7] Frenkel, H. S.: Recherches sur la fièvre aphteuse. Emploi d'épithélium lingual bovin importé de pays indemnes du virus de la fièvre aphteuse pour la culture *in vitro* de ce virus sur une échelle pratique. Bull. Off. internat. Epizoot., *39*, (1953): 131-139.

The Effects of Some Phenothiazine Derivatives and Analogs on the Free-Living Stages of Horse Strongyles

NORMAN D. LEVINE, Ph.D., and VIRGINIA IVENS, B.S.

Urbana, Illinois

SINCE phenothiazine was introduced as an anthelmintic by Harwood, Jerstad, and Swanson,[7] many investigations have been carried out on its effect against various nematodes. Phenothiazine is highly effective against some, less effective although still usable against others, and completely ineffective against many.

LITERATURE CITED

It was felt that some compounds related to phenothiazine might also be nematocidal and might be effective against some nematodes against which phenothiazine is valueless. Some work along this line was done soon after the discovery of phenothiazine. For example, Harwood, Habermann, and Guthrie[6] found that phenoxathiin (phenothioxin), xanthone, and phenazine were inactive against *Ascaris lumbricoides* in swine, and Gordon and Lipson[5] found that phenothiazone and thionol were ineffective, while phenoxathiin had little activity against *Haemonchus contortus* in sheep. Later, Gordon[4] added methylene blue, xanthone, and trimethylthianthrene to the list of compounds inactive against this parasite. However, Whitten[14] found that phenothiazine-5-oxide was active against *H. contortus* in sheep. Other reports are reviewed by Davey and Innes,[1] Edwards,[2] and Findlay.[3]

In the present paper, the effects of phenothiazine and 23 derivatives, analogs, and related compounds on horse strongyle larvae and/or eggs in feces were studied. Some of these results have been reported in abstract form by Levine and Ivens.[11] Parnell[12] had previously reported the effects of phenothiazine and phenoxathiin, Levine[8] that of phenothiazine, and Parnell and Mackie[13] those of phenothiazine and 12 derivatives. The only compounds reported on, both in the above papers and in the present one, are phenothiazine and phenoxathiin.

From the College of Veterinary Medicine and Agricultural Experiment Station, University of Illinois, Urbana.

Dr. Levine is professor, College of Veterinary Medicine and Agricultural Experiment Station, University of Illinois, Urbana. Miss Ivens is assistant in the College.

We acknowledge the cooperation of Dr. Henry Gilman, Department of Chemistry, Iowa State College, Ames, in supplying the 10-ethylphenothiazine, 10-ethylphenothiazine-5, 5-dioxide, and 10-ethylphenothiazine-5-oxide used in this study and of the Grasselli Chemicals Department, E. I. DuPont de Nemours and Co., in supplying the 10-acetylphenothiazine, 10-isobutyrylphenothiazine, 10-caprylylphenothiazine, 10-hendecanolylphenothiazine, 10-lauroylphenothiazine, ethyl and butyl esters of γ-oxo-10-phenothiazinebutyric acid, phenoxathiin-10, 10-dioxide, and xanthone.

MATERIALS AND METHODS

The technique used in this study has been described by Levine.[9,10] Briefly, the compound to be tested was premixed with talcum powder to give it body and then mixed with fresh horse feces containing strongyle eggs. The mixture was placed in a wide-mouthed glass jar, and the jar was placed in a dark cupboard at room temperature for a week. At the end of this time, the feces were examined for larvae, using the Baermann technique. If no larvae could be found, the compound was considered active. Untreated controls were also set up and examined. Each compound was set up in concentrations of 0.1, 0.05, 0.025, 0.01, and 0.005 M. If the compounds were effective at 0.005 M., further tests at lower concentrations were then carried out.

Well over 95 per cent of the eggs and larvae in the feces were those of small strongyles and the remainder were those of Strongylus spp.

RESULTS

Phenothiazine was active against horse strongyle larvae and/or eggs at a concentration of 0.025 M. (0.5%). Of the phenothiazine derivatives tested, 10-acetylphenothiazine, 10-caprylylphenothiazine, 10-hendecanolylphenothiazine, 10-lauroylphenothiazine, 10-isobutyrylphenothiazine, the ethyl ester of γ-oxo-10-phenothiazinebutyric acid, the butyl ester of γ-oxo-10-phenothiazinebutyric acid, 10-ethylphenothiazine, 10-ethylphenothiazine-5-oxide, and 10-ethylphenothiazine-5, 5-dioxide were inactive at 0.1 M.; while the tetrapotassium salt of 1, 3, 7, 9-phenothiazinetetrasulfonic acid, 5, 5-dioxide prevented the development of all larvae in feces at this concentration.

Of the phenothiazine analogs tested, phenoxathiin and chlorophenoxathiin prevented larval development at a concentration of 0.025 M. Phenoxathiin-10, 10-dioxide, however, was inactive at a concentration of 0.1 M. Xanthone and anthrone were inactive at a concentration of 0.1 M., while xanthydrol was active at 0.05 M. Phenazine and phenoxazine were active at 0.01 M., pyocyanin dihydrochloride was active at 0.005 M., but 2-aminophenazine was not active at 0.1 M.

Two aliphatic secondary amines which might perhaps have some stereochemical relationship to phenothiazine were also tested. These compounds, hexyl-*iso*-octyl amine and di-*n*-hexyl amine, were both toxic at a concentration of 0.01 M.

DISCUSSION

Certain conclusions can be drawn regarding the effect on toxicity of changes in the phenothiazine molecule. Substitution on the nitrogen atom destroys or greatly decreases its toxicity. This occurred with ten of the compounds tested: 10-acetylphenothiazine, 10-caprylylphenothiazine, 10-hendecanolylphenothiazine, 10-isobutyrylphenothiazine, 10-lauroylphenothiazine, the ethyl ester of γ-oxo-10-phenothiazinebutyric acid, the butyl ester of γ-oxo-10-phenothiazinebutyric acid, 10-ethylphenothiazine, 10-ethylphenothiazine-5-oxide, and 10-ethylphenothiazine-5, 5-dioxide. The tetrapotassium salt of 1, 3, 7, 9-phenothiazinetetrasulfonic acid, 5, 5-dioxide, which had no substituent on the nitrogen atom, was toxic at 0.1 M.

Replacement of the nitrogen atom of the phenothiazine molecule with oxygen produces the analog, phenoxathiin. This compound is as toxic as phenothiazine. Chlorination of phenoxathiin does not affect its toxicity. However, when 2 oxygen atoms are substituted on the sulfur atom of phenoxathiin, toxicity is destroyed or greatly decreased.

Replacement of the sulfur atom of phenothiazine by oxygen to form phenoxazine slightly increases the toxicity of the molecule. Replacement of the sulfur of phenothiazine with a carbonyl group and the nitrogen with an oxygen atom to form xanthone greatly decreases or destroys the toxicity of the molecule. However, if the carbonyl group of xanthone is reduced to the corresponding secondary alcohol to form xanthydrol, toxicity is partially restored. When the sulfur and nitrogen of phenothiazine are replaced by a carbonyl group and a methylene group, respectively, to form anthrone, the toxicity of the molecule is greatly decreased or destroyed.

When the sulfur of the phenothiazine molecule is replaced by nitrogen to form phenazine, toxicity is somewhat increased. Two phenazine rings form the basic structure of the pyocyanin dihydrochloride molecule; this compound is twice as toxic as phenazine.

Replacement of the phenyl groups of diphenyl amine, the parent compound of phenothiazine, by certain alkyl groups gives rise to compounds more toxic than phenothiazine. This is shown by the action di-*n*-hexyl amine and hexyl-*iso*-octyl amine. However, there is no evidence that the mode of action of these compounds is similar to that of phenothiazine.

SUMMARY

Phenothiazine and 23 related compounds were tested against horse strongyle larvae and eggs in feces. Phenothiazine itself killed or prevented the development of larvae in feces at a concentration of 0.025 M. (0.5 %). Only one of 11 derivatives, the tetrapotassium salt of 1, 3, 7, 9-phenothiazinetetrasulfonic acid, 5, 5-dioxide, was effective at 0.1 M., the highest concentration tested.

Of the analogs tested, phenoxathiin and chlorophenoxathiin were active at 0.025 M., pyocyanin dihydrochloride at 0.005 M., phenazine and phenoxazine at 0.01 M., and xanthydrol at 0.05 M. Phenoxathiin-10, 10-dioxide, xanthone, anthrone, and 2-aminophenazine were not effective at 0.1 M.

Two related aliphatic compounds, hexyl-*iso*-octyl amine and di-*n*-hexyl amine, were toxic at a concentration of 0.01 M.

Modifications of the phenothiazine molecule had varying effects on the toxicity of the compounds produced. Substitution on the nitrogen atom of phenothiazine decreased or destroyed its toxicity. Replacement of the nitrogen atom of phenothiazine by oxygen or of the sulfur by nitrogen did not decrease the toxicity of the compound. Replacement of one of the heterocyclic atoms of phenothiazine by a carbonyl group and the other by an oxygen atom or a methylene group destroyed or greatly reduced toxicity. In the former compound, reduction of the carbonyl to the corresponding secondary alcohol partially restored toxicity.

References

[1] Davey, D. G., and Innes, J. R. M.: The Present Position of Phenothiazine as an Anthelmintic. Vet. Bull. *12*, (1942): R7-R14.

[2] Edwards, J. T.: Phenothiazine in Veterinary Medicine; A Review of the Literature of 1942-1946. Imp. Agric. Bureaux, Weybridge, England, (1947): 36.

[3] Findlay, G. M.: Recent Advances in Chemotherapy.

3rd ed. Vol. 1, J. & A. Churchill, London, (1950): 124-150.

[4] Gordon, H. M.: The Anthelmintic Efficiency of Phenothiazine Sulphoxide Against *Oesophagostomum Columbianum* in Sheep. Austral. Vet. J., *24*, (1948): 335-336.

[5] Gordon, H. M., and Lipson, M.: Preliminary Examination of the Anthelmintic Efficiency of Certain Compounds Related to Phenothiazine. J. Council Sci. and Indust. Res. (Australia), *13*, (1940): 173-177.

[6] Harwood, P. D., Habermann, R. T., and Guthrie, J. E.: Studies of Phenothiazine and Related Substances as Anthelmintics. J. Parasitol. *26*, (1940): Suppl.: 15.

[7] Harwood, P. D., Jerstad, A. C., and Swanson, L. E.: The Efficacy of Phenothiazine for the Removal of Ascarids and Nodular Worms from Swine. J. Parasitol., *24*, (1938): 16-17.

[8] Levine, N. D.: The Effect of Various Compounds upon Horse Strongyle Larvae in Feces. Am. J. Vet. Res., *10*, (1949): 233-239.

[9] Levine, N. D.: The Use of Horse Strongyle Larvae in Screening Compounds for Anthelmintic Activity. Trans. Illinois Acad. Sci., *43*, (1950): 233-236.

[10] Levine, N. D.: Screening Tests of Iodine Compounds Against Horse Strongyle Larvae. Am. J. Vet. Res., *12*, (1951): 110-115.

[11] Levine, N. D., and Ivens, V.: The Effects of Some Phenothiazine Derivatives and Analogs on Horse Strongyle Developmental Stages in Feces. J. Parasitol., *39*, (1953): Suppl.: 36-37.

[12] Parnell, I. W.: The Comparative Value of Some Further Chemicals in the Treatment of Manure Against Sclerostoma Larvae. Canad. J. Comp. Med. and Vet. Sci., *5*, (1940): 76-77.

[13] Parnell, I. W., and Mackie, A.: Some Observations on the Lethal Effects of Various Chemicals Against the Free-Living Stages of Sclerostoma (Nematoda). Brit. J. Pharmacol. and Chemo., *7*, (1952): 509-533.

[14] Whitten, L. K.: The Anthelmintic Efficiency of Phenothiazine Sulphoxide Against *Haemonchus Contortus* and Certain Large Bowel Parasites of Sheep. Austral. Vet. J., *24*, (1948): 114-115.

The Negative Role of the Ewe in the Transmission of Vibriosis of Sheep

H. MARSH, D.V.M.; B. D. FIREHAMMER, M.S.; L. H. SCRIVNER, D.V.M.

Bozeman, Montana, and Moscow, Idaho

THE OBSERVED epizootiology of vibriosis of sheep and the experimental work on the transmission of the infection have not yet definitely established the primary avenue of infection and the means by which the disease is carried from one flock to another and from one breeding season to the next. The usual history has shown a sporadic occurrence with no discoverable source of the infection. There have been heavy losses for one or two seasons in many flocks, followed by years of apparent freedom from the disease. Observations over a period of years indicate that this disease does not become enzootic on affected ranches nor does it become epizootic.

In the study of the epizootiology of ovine vibriosis, there are several possibilities to be considered as the means of transmission between sheep and between flocks. One of these is the possible spread of the infection by carrier ewes.

Only three published reports of observations pertaining to this subject have been found. Baker and Stone[1] reported on 8 ewes which aborted in 1937 and lambed normally in 1938. Ryff[2] reported on a flock in which 28 of 79 ewes aborted in 1940 as the result of *Vibrio fetus* infection, and in 1941 there were no abortions in the same flock of ewes. Biswal, Rhoades, Barto, and Morrill[3] reported an outbreak in which 32 of 114 ewes aborted in 1952, and in 1953 there were no abortions in the flock.

Observations on ewes at the Montana Experiment Station indicate that aborting ewes lose the infection before the next breeding season. In the small flock maintained at the Veterinary Research Laboratory, 11 ewes which were experimentally infected, and 11 which developed vibriosis spontaneously, were returned to the flock and bred over periods varying from one to five years without evidence of vibriosis in these ewes or in the other ewes of the flock. In 49 possible pregnancies for the 22 ewes, following vibrionic abortion, there were 44 normal parturitions; each of 3 ewes was dry (failed to breed) once; there was one abortion not caused by vibriosis; and there was one case of dystocia and metritis in which *Vibrio fetus* was not involved. In the cases of 8 of the spontaneously infected ewes, vaginal swabs were taken after normal parturition the year following the abortions, and no vibrios were found.

Individual histories are also available on 146 ewes known to have aborted in the flock maintained by the Department of Animal Industry of the Montana Experiment Station. Of these 146 ewes, 136 lambed normally the year following abortion; 2 aborted but cultures from the fetuses failed to show the presence of *Vibrio fetus;* there were five stillbirths which were not investigated bacteriologically; 3 ewes were reported as dry. As these 146 aborters were removed from the flock and run separately during the year following abortion, we do not have evidence as to their possible carrier status based on the exposure of susceptible ewes, as we did in the case of the flock of the veterinary laboratory.

In 1952, Mr. J. E. Nordby, director of the U. S. Sheep Experiment Station at Dubois, Idaho, reported that 647 of 2,527 ewes aborted. About 500 of the aborting ewes were retained and bred again. They were distributed through the various breeding lots so that the exposure to these aborting ewes was general throughout the flock, but in 1953 there were no abortions which were thought to be attributable to vibriosis.

EXPERIMENTAL EXPOSURE OF SUSCEPTIBLE EWES TO EWES FROM AN INFECTED FLOCK DURING THE BREEDING SEASON AND GESTATION PERIOD

In October, 1952, ewes from the flock of the U. S. Sheep Experiment Station at Dubois, Idaho, where 26 per cent of the ewes bred had aborted

From the Montana Veterinary Research Laboratory (Marsh and Firehammer) (Montana Experiment Station and Livestock Sanitary Board, cooperating) and the Department of Veterinary Science (Scrivner) at the University of Idaho. Paper No. 324 journal series, Agricultural Experiment Station, Montana State College, Bozeman. Also approved by the director of the Idaho Agricultural Experiment Station as research paper No. 371.

The experiments reported here are phases of a regional attack on the sheep vibriosis problem in which research agencies in Colorado, Idaho, Montana, Utah, and Wyoming are participating.

in 1952, were used in two experiments designed to determine whether such ewes might act as carriers of infection. In one experiment at the Montana Veterinary Research Laboratory, ewes were used which were known to have aborted in 1952, and in the other experiment at the Idaho Experiment Station ewes were used from the same flock which had lambed normally in 1952.

Experimental Procedure.—Forty-two ewes of the flock of the U. S. Sheep Experiment Station which had aborted in 1952 were moved to Bozeman, Mont., on October 15 and placed with 50 yearling ewes from the flock of the Montana Experiment Station, in which there had been no known *Vibrio fetus* infection for nine years. The lot of 92 ewes was placed in a large corral which had been resurfaced to eliminate a possible premise infection. The water was supplied by the city system to eliminate a possible source of infection. Three rams from the flock of the experiment station were placed with this lot of ewes on November 14. The 42 previous aborters and the 50 yearlings were bred, wintered, and lambed as one lot.

On Nov. 18, 1952, blood samples were taken from all of the ewes in the experiment. The serums were tested for agglutinins with an antigen prepared from a strain of *Vibrio fetus* of the same serological type as the organism recovered from fetuses aborted at the U. S. Sheep Experiment Station in 1952. Three of the sheep from Dubois showed a titer of 1:25 and 2 showed a titer of 1:50. None of the test yearlings showed any significant reaction. The 3 rams to which the ewes were bred were tested for agglutinins before they were placed with the ewes. No significant titers were found.

A careful record was made of all lambs dropped. As soon as practicable after the lambs were dropped, vaginal swabs were made from the ewes which had aborted in 1952. The swab was passed in through a glass speculum to the region of the cervix. Smears were stained and examined, and cultures were made on serum-agar slants, in semisolid "hormone" agar, in semisolid thiol medium, and on thiol and serum-agar plates. The cultures were incubated in an atmosphere containing 15 per cent CO_2.

On October 6, 24 ewes (17 Columbia and 7 Targhee) from the flock of the U. S. Sheep Experiment Station, which had lambed normally in 1952, were moved to Moscow, Idaho. Ten young ewes from the Idaho station flock (8 lambs, 1 yearling, and 1 3-year-old) were bred with the ewes from Dubois, and 6 bred aged ewes were wintered with this group. Breeding of the Targhee ewes was started October 10, and 5 of the Idaho station ewes were added on October 21. None of the Columbia ewes were bred until November 5, when all the sheep were placed in one lot.

On Nov. 5, 1952, blood samples were taken from the 40 ewes in this experiment and from the 2 rams to which they were bred. The serums were tested for agglutinins, using an antigen prepared from the strain of *Vibrio fetus* recovered in 1952 from a fetus aborted at the U. S. Range Sheep Experiment Station. There was no significant agglutination, none of the serums showing complete agglutination at 1:25.

Vaginal swabs were made from each ewe at some time before parturition. Smears were prepared and cultures were made in Brewer's modified medium, tryptose agar, and serum or blood agar. After parturition, at a time varying from a few minutes to five days, the cervix, and in many cases the endometrium, were swabbed and smears were examined.

RESULTS

Of the 42 ewes which had aborted in 1952, 41 carried their lambs to full term with no evidence of infection of any kind. The remaining ewe in this lot aborted at about four months. On March 11, she did not eat normally and was depressed. There was evidence of a mucoid vaginal discharge. Her temperature was 103.5 F. Six days later she aborted. The temperature at that time was 101.5 F. There was no vaginal discharge. Smear from a vaginal swab showed a few organisms which somewhat resembled *Vibrio fetus*, but cultures failed to develop vibrios. The fetus was about 35 cm. long and had not yet developed any wool. It apparently had been dead for some time. Cultures from the heart, liver, and stomach of the fetus developed no bacteria, except for one culture which developed a diphtheroid. The ewe did not recover from the abortion, and on March 27 she was killed for bacteriological examination. The autopsy showed no pathology except in the uterus. Both horns of the uterus had been gravid. They both contained a creamy purulent material. Smears from this exudate showed gram-negative rods and a few gram-positive cocci but no vibrios. Cultures were made from the uterine exudate and from the heart blood. The heart cultures remained sterile. The cultures from the uterus developed a minute gram-negative coccobacillus and gram-positive cocci. It was concluded that this abortion was not produced by *Vibrio fetus* infection.

Of 37 vaginal swabs made at the time of parturition, 34 were definitely negative for the presence of *Vibrio fetus* as shown by smears and cultures. No organisms resembling vibrios were found in direct smears from any of the 37 swabs but, in cultures from the swabs from 3 ewes, slen-

der gram-negative rods showing some curving were found in mixed culture. These organisms showed some morphological resemblance to a Vibrio, but it was the opinion of the authors that they were not *Vibrio fetus*.

Attempts were made to isolate the organisms for pure culture study, but these attempts failed. The organism from ewe 1056-W showed more resemblance to *Vibrio fetus* than either of the others, and attempts to isolate this organism from the mixed culture were quite extensive. The organisms grew well in mixed culture in semisolid agar, but no growth on the surface of the plates was obtained, using several mediums and several variations of atmospheric conditions, from aerobic to anaerobic, and with increased CO_2 concentration. The differential effect of crystal violet in the medium was tried unsuccessfully. The mixed culture from 1056-W, containing a good percentage of the Vibrio-like organisms, was inoculated into pregnant guinea pigs. One of the guinea pigs aborted, but cultures from the fetus and from the uterus developed no Vibrio. These cultures developed a gram-negative coccobacillus in pure culture.

In order to obtain additional information in regard to the bacteriological status of the genital tract of ewe 1056-W, a laparotomy was performed ten days after parturition. The uterus was still turgid, and involution was not completed. The uterus was opened and found to contain a considerable amount of bloody degenerating material. Three degenerating cotyledons were removed and dropped into broth culture medium. Smears and a hanging drop preparation from this material were negative for vibrios but showed some cocci. Culture from this material developed only micrococci.

It was concluded that the organism which somewhat resembled *Vibrio fetus* in the cultures from swabs from ewe 1056-W was not *Vibrio fetus* and that this ewe showed no evidence of being a carrier of *Vibrio fetus*.

Further information on the bacteriology of the genital organs of the ewes, which had aborted in 1952, was available when 2 of them died from exposure after shearing, death occurring six weeks after parturition. Smears and cultures from the uterus, cervix, and vagina were negative for Vibrio. The material for the smears and cultures was prepared by scraping the mucosa and adding the scraping to a tube of broth. Cultures were made from this broth suspension on serum-hormone agar slants and plates, on thiol plates, and in semisolid serum-agar, with incubation in a 15 per cent CO_2 atmosphere.

Of the 50 yearling ewes which were bred and wintered with the 42 ewes which aborted in 1952, 1 was dry and 49 produced living lambs at full time, with no evidence of the presence of any pathological condition, except dystocia in 2 ewes.

On March 17, 1953, about one month before lambing started, all the sheep in the experiment were tested for agglutinins in the blood serum. On this test, 4 of the 5 sheep from Dubois showing titers of 1:25 or 1:50 in November had lost their reactions, while 1 which had a titer of 1:50 in November retained the same titer. Three ewes which had been negative in November showed the low titer of 1:25 in March. One ewe which had been negative in November had developed a titer of 1:100. This ewe lambed normally, and vaginal swabs were negative for *Vibrio fetus*. Four of the 50 test yearlings showed low titers in March, 3 at 1:25 and 1 at 1:50. The significance of these slight reactions in the yearlings is not known.

Of the 24 ewes from Dubois which lambed normally in 1952, 21 lambed normally in 1953, 2 were dry and 1 gave birth to a small, weak lamb. No *Vibrio fetus* was found in the lamb or its mother. One of the Idaho station ewes aborted twins after developing pregnancy disease. The result of a bacteriological examination of this ewe and the lambs was negative. All the other exposed ewes lambed normally except 1 which was dry.

All the smears and cultures from the vaginal and uterine swabs were negative for *Vibrio fetus*.

Blood samples were taken from all the ewes after parturition, and no significant agglutinins were found.

DISCUSSION

The experiments reported here confirm the conclusion from observations on the carrier ewe problem at Montana State College and at the U. S. Sheep Experiment

Station, as well as general field observations over a period of thirty years, that infected ewes do not retain the infection from one season to the next. The 92 ewes in the first experiment carried 120 lambs to full term. Of these 120 lambs, 116 were born alive. Of the 4 stillbirths, 2 were a pair of twins in a dystocia case, and the other 2 were twins of living lambs.

Although there was one abortion in the previously infected lot of ewes, *Vibrio fetus* was not found in the genital tract. This is in accord with previous observations that some ewes which abort as the result of vibriosis may abort and show metritis the following season as the result of a secondary infection with organisms other than *Vibrio fetus.*

It was also concluded that the ewes which had aborted in 1952 were not carriers of the infection in 1953. This conclusion was reached even though smears or cultures from the vaginas of 4 ewes showed some organisms in mixed culture which showed some resemblance to *Vibrio fetus.*

The second experiment, in which nonaborters from an infected flock were involved, adds additional evidence that ewes from this flock did not carry the infection into the next breeding season.

SUMMARY

Observations in three experimental flocks of sheep, and in the field, have indicated that ewes infected with *Vibrio fetus* do not carry the infection to the next breeding season.

An experiment is reported in which 50 yearling ewes from a flock which had been free from known vibriosis for nine years were bred, wintered, and lambed with 42 ewes all of which had aborted as the result of vibriosis during the previous season. Vibriosis did not develop in any of the exposed yearlings, and no evidence of the presence of *Vibrio fetus* could be found in the previously infected ewes.

In another experiment, 10 ewes from a flock presumably free of vibriosis were bred, wintered, and lambed with 24 ewes from an infected flock which had lambed normally in the previous season. No evidence of the presence of *Vibrio fetus* could be found.

References

[1] Baker, D. W., and Stone, W. S.: A Study of *Spirillum Ovis* Infection in a Group of Ewes Maintained Under Observation for Two Successive Lambing Seasons. Cornell Vet., *29*, (1939): 32-34.

[2] Ryff, J. F.: Failure of Natural *Vibrio Fetus* Infection to Carry Over in Ewes. Am. J. Vet. Res., *2*, (1941): 367-368.

[3] Biswal, G., Rhoades, H. E., Barto, P. B., and Morrill, C. C.: Observations of an Outbreak of Vibriosis in Sheep. J.A.V.M.A., *123*, (1953): 410-414.

Preliminary Studies of Parasitism in Sheep on Rangelands

N. F. BAKER, D.V.M.; W. M. LONGHURST, Ph.D.; D. T. TORELL, M.S.; W. C. WEIR, Ph.D.

Davis, California

A LARGE portion of the north coastal region of California consists of rangelands which are unsuitable for irrigation, cultivation, or the production of timber. These areas are used to a large extent for the production of feeder lambs. Compared with irrigated pastures, these ranges provide sheep with a relatively low plane of nutrition, and lambs raised on them consequently have a slower rate of growth.

As these hill ranges become dry, usually during the months of May and June, the quality of the forage drops off rapidly and all lambs not kept over as replacements are either moved to irrigated pastures or sent to market.

The quality of lambs raised on these areas is undoubtedly largely a reflection of the range condition as it affects them both directly and indirectly through effects on the ewes. However, there remains the possibility that there may be other interrelated or additive factors. The most commonly encountered problem of this nature is parasitism.

Longhurst and Douglas [4] have shown that parasitism in lambs could well be a problem in this area and have suggested the possible relationship between nutrition and parasitism. Whitlock [7,8] has summarized the results of others and cited experimental evidence which indicates "that the trichostrongylidoses are commonly complex diseases in which the parasite plays a role of secondary etiological significance." He also suggests that the nutrition of the lamb, both prenatal and postnatal, as influenced by the nutrition of the ewe, is an important factor in the development of trichostrongylidoses. Stewart and Crofton [6] indicated that in a mineral-deficient area of Great Britain, the effects of the mineral deficiency and parasitism had no appreciable interaction but were independent and additive.

MATERIALS AND METHODS

The study was conducted at the Hopland Field Station of the College of Agriculture of the University of California located in southeastern Mendocino County. The field station covers an area of nearly 4,700 acres, ranging in elevation from 500 to 3,000 ft. Vegetation is typical of the region, consisting of a grass and oak woodland association, interspersed with patches of chaparral.

Precipitation follows a Mediterranean pattern and occurs largely from October through April, with new grass and herbaceous vegetation appearing in November. The annual rainfall averages about 35 in. During the year 1952-1953, when this work was conducted, the total rainfall was 49 in. Winters are not severe in this area and green feed is available into May, when forage begins to dry, except for small areas of seepage around springs.

In this experiment, 123 white-faced ewe lambs and 20 white-faced wether lambs were used. These lambs were all born on the Hopland Field Station during the months of December, 1952, and January, 1953.

At marking time, Feb. 2, 1953, all of the ewe lambs were weighed and separated into two groups, a control group of 63 and a "treated" group of 60 lambs. At the same time, 20 wether lambs were selected and 10 were added to each of the control and the treated groups. The lambs were divided on the basis of weight so that the average weight of the two groups would be nearly the same but no attempt was made to conduct pair trials. All lambs in the two treated groups were then drenched with 12.5 Gm. of phenothiazine and turned free on the range.

Four wether lambs, 2 from each group, were autopsied each month from March through July. All nematodes in the gastrointestinal tract were collected and counted. The parasites were then preserved in 70 per cent alcohol for identification.

At shearing time, eighty days later, all lambs were again weighed, rebranded, and those in the treated groups again drenched with 12.5 Gm. of phenothiazine. On the 126th day of the experiment, the lambs were weaned and those in the treated group were given 18.75 Gm. of phenothiazine each. On the 147th day, the lambs in the treated group were drenched with 25 Gm. of phenothiazine and

TABLE 1—Average Weight and Net Gains of Ewe Lambs

Controls	Start	80 days	126 days	147 days	172 days on supplement	172 days unsupplement.
Ave. weight	20.2	55.6	69.0	74.8	76.9	70.6
Net gain		35.4	13.4	5.8	2.1	—4.2
Treated						
Ave. weight	21.2	56.7	68.7	75.8	77.1	71.3
Net gain		35.5	12.0	7.1	1.3	—4.5

From the Agricultural Experiment Station, University of California, Davis; Dr. Baker, junior parasitologist, Dr. Longhurst, specialist; Mr. Torell, assistant specialist; Dr. Weir, assistant professor of animal husband y.

the ewe lambs were divided into four groups, two control and two treated. One control group of 33 lambs and one treated group of 30 lambs, as well as the remaining wether lambs, were placed on a supplemental free choice ration of 20 per cent salt, 40 per cent barley, and 40 per cent cottonseed meal. The other two groups, 30 control and 29 treated lambs, were kept on the range with no supplement.

The experiment was terminated on July 24, 1953, 172 days after it began.

EXPERIMENTAL RESULTS

Growth of Treated and Untreated Ewe Lambs.—The average weight of the 63 control lambs at the beginning of the trial was 20.2 lb. compared to 21.2 lb. for the 60 treated animals. Table 1 gives the summation of the average weights and net gains of the two groups. The net gain for the first 147 days, when half were placed on a supplemental ration, was identical in the two groups, 54.6 lb. During the next twenty-five days, the control and treated lambs on supplement made a net gain of 2.1 and 1.3 lb., while those with no supplement lost an average of 4.2 and 4.5 lb., respectively.

The differences in gain are insignificant, except when the supplemented lambs are compared to the unsupplemented animals.

Therefore, no beneficial effect on growth rate can be attributed to the phenothiazine as used in this experiment. This could be due to one of two factors or to a combination of the two; either the worm burden was insufficient to retard the growth of the control lambs or the phenothiazine did not remove sufficient numbers of these parasites to allow increased gains in the treated lambs.

Total Number of Worms Present in Wether Lambs at Autopsy.—The number of worms present in the individual animals of any one group is extremely variable (table 2). This may be a reflection of individual variation in natural resistance, variation in exposure, a more marked and rapidly formed acquired immunity, or possibly a variation in the amount of nourishment which the lambs obtained from their dams. These possible variations emphasize the necessity of using large numbers of animals in experiments involving total worm counts, since the use of small numbers may lead to inaccurate conclusions.

It is concluded that the difference in the total number of worms occurring in control and treated animals is due primarily to the individual variation in members of the two groups and is not significant. It must be further concluded, therefore, that the phenothiazine therapy was of no value.

Apparently, parasites inhabiting the large intestine occur only in small numbers and for practical purposes none was found prior to June. What significance this may have in regard to the bionomics of these parasites can only be conjectured at this time.

Genera and Species of Parasites Encountered in the Abomasum of the Wethers.—The number of different species

TABLE 2—Total Number of Worms in the Gastrointestinal Tract of Autopsied Lambs

Eartag	Autopsy (date)	Abomasum	Ave. for month	Small intestine	Ave. for month	Large intestine	Ave. for month
			CONTROL WETHER LAMBS				
.2456	3-18-53	824		358		....	
.2458	3-19-53	1,052	938.0	191	274.5	....	
.2436	4-16-53	221		352		....	
.2431	4-18-53	1,106	663.5	381	367.0	....	
.2470	5-11-53	789		403		....	
.2445	5-26-53	564	676.5	704	553.5	....	
.2455	6-23-53	7		111		....	
.2485	6-29-53	321	145.5	602	356.5	11	5.5
.2472	7- 7-53	261		605		1	
.2441	7-24-53	353	307.0	1,757	1,181.0	2	1.5
			TREATED WETHER LAMBS				
.2446	3- 3-53	124		4		....	
.2466	3-21-53	681	402.5	84	44.0	1	0.5
.2454	4- 9-53	681		315		2	
.2443	4- 9-53	630	655.5	63	189.0	....	1.0
.2434	5-11-53	950		367		....	
.2433	5-22-53	61	505.5	142	254.5	....	
.2427	6-22-53	1,246		625		....	
.2438	6-25-53	468	857.0	310	467.5	....	
.2463	7- 6-53	12		808		3	
.2462	7-24-53	184	98.0	291	548.5	3	3.0

TABLE 3—Species of Nematodes in the Abomasum

Percentage of total worms by genus			Percentage species representing genera		
Genus	Control	Treated	Species	Control	Treated
Ostertagia	96.0	98.2	*O. circumcincta* males + all females	97.5	97.9
Trichostrongylus	4.0	1.5	*O. trifurcata* males	2.5	2.1
Haemonchus		0.5	*T. vitrinus* males	6.9	2.8
Cooperia		0.2	*T. colubriformis* males	2.7	
Nematodirus		0.2	Trichostrongylus spp. females	90.4	97.2

occurring in the abomasum was rather limited, five genera and seven species (table 3). Members of the genus Ostertagia were the most numerous and represented an average of 96.0 and 98.2 per cent of the total worms in the control and treated groups, respectively. *Ostertagia circumcincta* was the most common species, the females and males combined comprising 97.5 and 97.9 per cent, while *O. trifurcata* males comprise the other 2.5 and 2.1 per cent of this genus. The ratio of females to males in this genus was of the order of 54 to 46.

Members of the genus Trichostrongylus were second in number and averaged 4.0 and 1.5 per cent of the nematode burden in the control and treated animals, respectively. This genus was broken down into *T. vitrinus* males, *T. colubriformis* males, and Trichostrongylus spp. females. The average percentages for the control animals were 6.9, 2.7, and 90.4, while for the treated animals they were 2.8, 0.0, and 97.2, respectively. The marked unbalance of the sex ratio in this genus is of interest.

Other genera and species found were *Haemonchus contortus, Cooperia oncophora,* and *Nematodirus fillicolis.* All but the first of these were considered to be incidental and probably the result of collection procedures. *Haemonchus contortus* is apparently unable to perpetuate itself on this range except in limited numbers. During the studies by Longhurst and Douglas,[4] this parasite was introduced to the Hopland ranges with sheep brought in from the Sacramento Valley but spread to only a few animals.

There was no apparent seasonal variation in genera or species during the course of this study nor were any changes in the sex ratios noticed.

Genera and Species of Parasites Encountered in the Small Intestine. — The nematodes inhibiting the small intestine were found to represent four genera and six species (table 4). The most common genus in the control animals was Trichostrongylus, which accounts for 54.3 per cent of the parasites present. This genus was represented in these animals as follows: *Trichostrongylus vitrinus* males, 48.4 per cent; *T. colubriformis* males, 3.7 per cent; and Trichostrongylus spp. females, 47.9 per cent. In the treated animals, the genus Trichostrongylus accounted for 42 per cent and were identified as *T. vitrinus* males, 45.2 per cent; *T. colubriformis* males, 2.5 per cent; and Trichostrongylus spp. females, 52.3 per cent. The sex ratio of this genus in the small intestine was much more equal than in the abomasum.

The next most common genus encountered in the small intestine was Nematodirus, which accounted for 45.7 per cent parasites in control lambs. This genus was further classified as *Nematodirus spathiger* females, *N. fillicolis* females, Nematodirus spp. males, and Nematodirus spp. immature females; the percentages in control lambs were 12.0, 27.7, 32.0, and 27.6, respectively. In the treated lambs, the genus Nematodi-

TABLE 4—Species of Nematodes in the Small Intestine

Percentage of total worms by genus			Percentage species representing genera		
Genus	Control	Treated	Species	Control	Treated
Trichostrongylus	54.3	42.0	*T. vitrinus* males	48.4	45.2
Nematodirus	45.7	56.4	*T. colubriformis* males	3.7	2.5
Ostertagia		1.6	Trichostrongylus spp. females	47.9	52.3
Cooperia	0.03		*N. spathiger* females	12.0	10.9
			N. fillicolis females	27.7	28.7
			Nematodirus spp. immature females	27.6	24.7
			Nematodirus spp. males	32.0	35.7

rus accounted for 56.4 per cent consisting of *N. spathiger* females, 10.9 per cent; *N. filicollis* females, 28.7 per cent; Nematodirus spp. males, 35.7 per cent, and Nematodirus spp. immature females, 24.7 per cent.

No specific trends concerning either of the foregoing genera were revealed in the data. Special attention was given to the immature worms of the genus Nematodirus but the only conclusion reached was that the lambs must have been obtaining infective larvae in relatively large numbers as late as July, when the only source of moisture was in small areas surrounding springs.

Trichostrongylus spp. and Nematodirus spp. accounted for 100 per cent and 98.4 per cent of the worms in the control and treated lambs, respectively. In 1 control lamb a small number of Cooperia spp. was found, or an average of 0.03 per cent per lamb. In the treated animals, Ostertagia spp. accounted for 1.6 per cent of the worms. In addition to nematodes, many of the lambs had light infections of *Moniezia expansa*.

DISCUSSION

The numbers of nematodes which reached adult stages were relatively small, averaging approximately 527 in the abomasum and 424 in the small intestine and, on a nutritional plane such as may be found on irrigated pastures, they would probably be of no consequence. However, the fact that the animals were on a declining nutritional level and that the protein and available energy were decreasing during the latter months of spring suggests that parasitism might still be a factor in the production of feeder lambs on this range.

The individual lambs varied greatly in total parasitism but the relative percentage of species was rather constant. This would indicate that the resistance was to parasitism as a whole and not resistance acquired by exposure to species of parasites. The wide variation encountered in parasite numbers is believed to be due to natural resistance and possibly to nutrition rather than to acquired immunity.

The species *Ostertagia circumcincta* is by far the most common parasite in the abomasum of lambs raised on these ranges, as well as in more productive irrigated pastures of California. Species of Trichostrongylus and Nematodirus are about equal in number in the small intestine. *Trichostrongylus colubriformis* was the predominant species of this genus while *Nematodirus filicollis* predominated over *N. spathiger*.

It appears that Ostertagia spp., Trichostrongylus spp., and Nematodirus spp. are much more adapted to the type of environment present in the Hopland Field Station than are the other genera encountered. Oesophagostomum spp. and *Chabertia ovina* are very common parasites of sheep on adjoining, lower, irrigated pasture lands. *Haemonchus contortus* and Cooperia spp., while not numerous on these adjoining irrigated pastures, are nevertheless able to propagate themselves.

The fact that the phenothiazine was ineffective as used in this experiment makes it impossible to determine the relationship between parasitism and nutrition or to determine the value of medication as used.

There is no definite explanation for the apparent failure of phenothiazine to remove appreciable numbers of nematodes. Several factors may have played a part: (1) It is possible the dosage was too low; (2) the interval between drenches may have been too long (an explanation only if it is assumed that there is a maximum number of worms able to infest a lamb and that in the treated lambs this point was reached between drenching and sampling, which does not seem probable); (3) the phenothiazine may have been relatively ineffective; or (4) the nematodes might have developed resistance to phenothiazine.

TABLE 5—Distribution of Phenothiazine Particle Sizes

Photomicrograph	Total particles	0 to 17 μ		17 to 33 μ		33 to 50 μ		50 to 67 μ		67 μ	
		(No.)	(%)	(No.)	(%)	(No.)	(%)	(No.)	(%)	(No.)	(%)
1	115	43	37.4	51	44.3	9	7.8	6	5.2	6	5.2
2	137	55	40.1	56	40.8	7	5.1	9	6.6	10	7.3
3	108	55	50.9	38	35.2	5	4.6	5	4.6	5	4.6
4	128	42	32.8	39	30.5	25	19.5	7	5.5	15	11.7
5	87	22	25.3	37	42.5	14	16.1	7	8.0	7	8.0
6	126	56	44.4	40	31.8	6	4.8	8	6.3	16	12.7
Ave.	117	45.5	38.9	43.5	37.2	11	9.4	7	6.0	10	8.5

The authors are fully aware that in the first two drenchings, and possibly the third, the dosage was below that of the usually recommended levels. The Seventh National Formulary, and many writers, recommend the use of 15 Gm. for lambs under 60 lb. in weight and 25 Gm. for those over 60 lb.

Even though the dosage was minimal, it is still impossible to conceive that it would have been so totally ineffective if the phenothiazine used had been effective. To aid in clarifying this point, 2 additional control lambs were treated with 50 Gm. of phenothiazine and autopsied in August. Worm counts were as follows: in the abomasum, 41 and 172, in the small intestine, 407 and 642. It is concluded, therefore, that the phenothiazine used was either relatively ineffective or the nematodes were resistant. Drug-resistant strains have been suspected but could not be confirmed.[5]

In recent outbreaks of helminthosis, which occurred in eastern Australia, it was found necessary to double the recommended dosage of phenothiazine to obtain satisfactory control of nematodes.[2] Evidence which subsequently accumulated pointed to the fact that commercial preparations of phenothiazine varied greatly in particle size. Gordon [1] had already reported that of two preparations, the one having the finer particle size was the more effective against *Oesophagostomum columbianum*. Gordon [3] has recently conducted trials involving several preparations of various particle sizes and has shown that an inverse relationship of particle size and anthelminthic efficiency exists. He concludes that although an optimum particle size has not been determined, preparations should not contain particles greater than 30 μ in diameter and a high proportion should be less than 20 μ. He believes that the larger particle size resulted in the requirement for the higher dosage.

The particle sizes of the phenothiazine used in this work were approximated by diluting a small portion of a well-mixed suspension and pipetting it onto a microslide. Six photomicrographs were then taken of different fields. The pictures were enlarged to facilitate measurement and each particle was measured. The size distribution of 701 particles measured in six fields selected at random are shown in table 5.

Examination of table 5 reveals that 23.9 per cent of the particles of phenothiazine used were over 33 μ in diameter while only slightly more than 38.9 per cent were below 20 μ. If relative volumes were calculated, well over 50 per cent of the total phenothiazine, on a weight basis, would be represented by particles of over 33 μ. Although the optimum particle size for maximum efficiency is not established, the writers believe that the high percentage of large particles present in the phenothiazine preparation used could account for the ineffectiveness of the dosage employed.

SUMMARY

A study of parasitism as it occurs on the Hopland Field Station of the University of California is reported.

Ostertagia circumcincta is the most common stomach worm in this area while *Trichostrongylus vitrinus*, *Nematodirus filicolis*, and *Nematodirus spathiger* are the most common parasites of the small intestine. Individual lambs showed a very marked difference in total worm counts but the percentage of species and sexes was surprisingly constant.

Phenothiazine as employed in this experiment was totally ineffective against the nematodes present in the gastrointestinal tracts of lambs. In view of the evidence presented, the writers conclude that this was due possibly to the high percentage of large particle sizes in the phenothiazine used.

References

[1] Gordon, H. Mcl.: Studies on Phenothiazine as an Anthelmintic. A Comparison of the Efficiency of Finely and Coarsely Ground Phenothiazine Against *Oesophagostomum Columbianum*. J. Council Scient. Indust. Res., Australia, *13*, (1940): 85-86.

[2] Gordon, H. Mcl.: The Wet Season and Worms in Sheep. Agric. Gaz., New South Wales, *61*, 1950: 445-447.

[3] Gordon, H. Mcl.: Personal communication. 1953.

[4] Longhurst, W. M., and Douglas, J. R.: Parasite Interrelationships of Domestic Sheep and Columbian Black-Tailed Deer. Tr. 18th North Am. Wildlife Conference, (1953): 169-188.

[5] Sinclair, D. P.: An Unsuccessful Attempt to Produce Resistance to Phenothiazine in *Trichostronglyus Colubriformis* of Sheep. Austral. Vet. J., *29*, (1953): 13-17.

[6] Stewart, W. H., and Crofton, H. D.: Parasitic Gastritis in Sheep. Field Trials with Phenothiazine and Mineral Supplement. Vet. Rec., *55*, (1943): 3-4.

[7] Whitlock, J. H.: The Relationship of Nutrition to the Development of the Trichostrongylidoses. Cornell Vet., *39*, (1949): 146-182.

[8] Whitlock, J. H.: The Relationship of the Available Natural Milk Supply to the Production of the Trichostrongylidoses in Sheep. Cornell Vet., *41*, (1951): 299-311.

A Comparison of *Clostridium Perfringens Epsilon* Toxin-Toxoid and Prototoxin-Toxoid in the Immunization of Young Lambs

LOUIS DS. SMITH, Ph.D., and TATS MATSUOKA, B.A.

Bozeman, Montana

THE *epsilon* toxin of type D *Clostridium perfringens* is accepted as being responsible for death of lambs due to enterotoxemia. This toxin is not produced as such by the bacteria but, rather, as a relatively inactive prototoxin which is converted into the active toxin by digestion with proteolytic enzymes. Although both the prototoxin and the toxin are antigenic, it does not seem to be generally recognized that a higher degree of immunity may be induced against the *epsilon* toxin when the material to be used as the immunizing agent has been treated with trypsin. Batty and Glenny [1] showed that guinea pigs, rabbits, and horses produced a higher level of antitoxin when immunized with trypsinized filtrates of type D *Cl. perfringens* than they did when filtrates not treated with trypsin were used. These authors mentioned that similar results had been obtained in lambs, although no details were given.

Smith and Marsh [2] were unable to obtain satisfactory immunity in lambs under 30 days of age with a commercial type D *Cl. perfringens* bacterin, although this product was known to produce a satisfactory state of immunity in lambs of feedlot age. Not only was a comparatively low titer of antitoxin obtained in the young lambs, but a number of them did not respond with a detectable level of antitoxin.* Since it is desirable to produce active immunity in young lambs, this investigation has been extended to include a comparison of the immunizing abilities of toxoids prepared from *epsilon* toxin and from *epsilon* prototoxin.

MATERIALS AND METHODS

The toxoids were prepared by growing type D *Cl. perfringens* in a broth medium composed of 3.0 per cent peptone, 1.0 per cent glucose, and 0.5 per cent dipotassium hydrogen phosphate, adjusted to pH 7.3. After active growth had started, 5 N sodium hydroxide was added every fifteen minutes to keep the pH between 8.0 and 8.5. When growth ceased in about five hours as judged by the cessation of gas formation and acid production, the culture was centrifuged and the supernatant fluid removed. To half of this fluid, U.S.P. trypsin was added to a final concentration of 0.25 per cent and it was held at 39 C. for one hour. Before trypsin treatment, the culture fluid contained about 30 mouse lethal doses per milliliter and, after trypsin treatment, it contained about 20,000 mouse lethal doses per milliliter. If the prototoxin is assumed to be nontoxic, then 99.85 per cent of the toxin was present as prototoxin in the portion not treated with trypsin. Formalin (0.5 %) was added to both portions and they were stored for seven days at 37 C. Potassium alum was added in the form of 10 per cent solution to a final concentration of 1.0 per cent. The formolized, alum-precipitated culture fluid which had not been treated with trypsin will be referred to as "prototoxin-toxoid"; that which had been treated with trypsin before formolization and alum precipitation will be referred to as "toxin-toxoid."

In the first experiment, 30 lambs, about 15 days of age were used. Ten were injected subcutaneously with 3.0 ml. of the prototoxin-toxoid and 20 with 3.0 ml. of the toxin-toxoid. Two lambs in the latter group died during the course of the experiment because of exposure to inclement weather. The lambs were bled three and four weeks later and the serums were tested for antitoxin. Eighteen weeks after the first injection, all of the lambs receiving the prototoxin-toxoid and 10 of the lambs receiving the toxin-toxoid were bled again and injected subcutaneously with 3.0 ml. of toxin-toxoid. Another bleeding was taken one week after the second injection.

Another group of lambs 5½ to 6 months of age was injected subcutaneously with 3 ml. of toxin-toxoid and was bled four weeks later. This group was included in order to determine the response of lambs of feedlot age to this antigen.

The serums were titrated for antitoxin by the method described by Smith and Marsh,[2] except that the dried *epsilon* toxin was dissolved in 60 per cent glycerin to give a concentration of 1 mg. per milliliter and this was used to make up the solutions of toxin for titrations twice each day. Serum-toxin mixtures were always injected within one hour of the time that they were mixed. The

From the Montana Veterinary Research Laboratory (Montana Experiment Station and Livestock Sanitary Board cooperating), Agricultural Experiment Station, Montana State College, Bozeman. Journal series paper No. 326.

This work was supported in part by a contract with the Office of Naval Research.

* The immunizing agent used by these workers had not been treated with trypsin. Their paper is in error on this point.

results of these titrations are expressed in the unitage adopted by the U. S. Bureau of Animal Industry: One BAI unit equals approximately 0.1 Burroughs-Wellcome unit or 0.075 Australian unit.

RESULTS AND DISCUSSION

From the results of the titrations of the four-week bleedings shown in table 1, it can be seen that the antigenic potency of the toxin-toxoid was appreciably greater that that of the prototoxin-toxoid. Since the antitoxin titers at the three- and four-week bleedings were quite similar, only the latter are shown. The difference between the average titers obtained with these groups at the four-week bleeding is 6.67 $\pm$ 1.64 units per milliliter. Of equal significance is the fact that all the lambs injected with the toxin-toxoid responded with a measurable antitoxin level, while 2 of the 10 injected with the prototoxin-toxoid did not. The serums found to contain antitoxin in the preliminary bleeding probably represent an immune state in the ewes.

The single lambs in the toxin-toxoid group showed a higher titer than did the twins, 11.38 units per milliliter compared to 4.87, a difference of 6.51 $\pm$ 3.27. This greater response by the single lambs may be due to the higher plane of nutrition or to a more advanced state of physiological maturity. Single lambs are much more susceptible to enterotoxemia than are twin lambs and, in this instance, the animals most needing immune protection may be those most easily able to produce it.

TABLE 1—The Titer of Antitoxin* of 15-Day-Old Lambs Immunized with Prototoxin-Toxoid and Toxin-Toxoid

Lamb No.	Twin or single	Preliminary bleeding	4 Weeks after first inject.	18 Weeks after first inject.	1 Week after second inject.
		PROTOTOXIN-TOXOID			
256	T	0.0	1.0	0.26	67.0
259	S	0.0	2.0	2.0	33.0
260	S	0.0	1.0	1.0	67.0
264	T	0.26	4.0	0.52	67.0
267	T	0.0	0.0	0.0	33.0
269	T	0.0	0.0	0.0	17.0
270	S	0.0	0.26	0.0	8.0
272	S	0.0	1.0	4.0	133.0
276	T	0.0	1.0	0.26	67.0
279	S	0.26	0.52	2.0	33.0
Mean titers			1.11	1.03	52.0
		TOXIN-TOXOID			
236	S	1.0	1.0	1.0	133.0
246	S	0.0	33.0	17.0	266.0
247	T	1.0	4.0	2.0	266.0
248	T	0.52	8.0	17.0	1065.0
251	S	0.0	2.0	1.0	1065.0
253	T	0.0	2.0	8.0	266.0
255	T	0.0	1.0	0.52	133.0
265	T	0.0	17.0	1.0	133.0
268	T	0.0	8.0	4.0	2130.0
271	S	0.0	0.26	0.26	8.0
252	T	0.0	1.0	....	....
257	T	0.0	2.0	....	....
261	S	0.0	0.52	....	....
262	S	0.0	4.0	....	....
263	S	0.52	17.0	....	....
266	T	0.0	4.0	....	....
275	T	0.0	0.52	....	....
280	S	0.0	33.0	....	....
Mean titers			7.77	5.24	543.6

* Antitoxin titer of serum in BAI units per milliliter.

The data obtained from the titration of serums collected eighteen weeks after the first injection indicate that the antitoxin level held up surprisingly well, particularly in the lambs immunized with the toxin-toxoid. Two lambs in each group (272, 279, 248, and 253) may have been naturally exposed to contact with type D *Cl. perfringens*, for the antitoxin level of their serums rose appreciably between the fourth and the eighteenth week after the first injection. It is possible that the lambs which had been injected with the toxin-toxoid would not need additional immunization before going into a feedlot. Further evidence that the toxin-toxoid induced a higher state of immunity than the prototoxin-toxoid is indicated by the greater response in antitoxin resulting from the injection of toxin-toxoid into both groups at 18 weeks of age. The mean antitoxin level of the protoxin-toxoid group rose from 1.03 to 52.0 units per milliliter and that of the toxin-toxoid group rose from 5.24 to 543.6 units per milliliter.

When the toxin-toxoid was tested in lambs of feedlot age, the results given in

TABLE 2—The Titer of Antitoxin* of Lambs of Feedlot Age Immunized with Toxin-Toxoid

Lamb No.	Preliminary bleeding	4 Weeks after injection
202	0.0	2.0
214	0.0	2.0
233	0.0	66.0
277	0.0	4.0
292	4.0	66.0
321	0.0	1.0
324	0.0	4.0
358	0.0	4.0
379	0.0	17.0
Mean titer		18.5

* Antitoxin titer of serum in BAI units per milliliter.

table 2 were obtained. Lamb 292 apparently had been naturally exposed to type D *Cl. perfringens* for the preliminary bleeding showed an appreciable antitoxin content in the serum. The animals in this group responded more uniformly than did those injected at 15 days of age. It is not known whether the antitoxin level at the time of exposure or the immune state which allows rapid production of antitoxin when the *epsilon* toxin is encountered is the more important for protecting animals in the field. However, it has been estimated that 0.5 to 1.0 unit of antitoxin per milliliter of serum is sufficient to protect lambs against clinical enterotoxemia. From the results of these experiments, it appears that immunization with toxin-toxoid such as used here will suffice to protect almost all of the lambs so immunized. It is to be expected, however, that a period of two or three weeks would elapse between the injection of the toxoid and the attainment of immunity.

SUMMARY

Lambs 15 days old were immunized with toxoids prepared from *Clostridium perfringens epsilon* prototoxin and toxin. The immunity obtained, as shown by the immediate response and by the response to a second injection, indicated that the toxin-toxoid was a better immunizing agent than prototoxin-toxoid. A satisfactory level of immunity was also obtained in lambs of feedlot age.

References

[1] Batty, I., and Glenny, A. T.: The Antigenic Efficiency of *Clostridium Welchii Epsilon* Toxin and Toxoid after Treatment with Trypsin. Brit. J. Exptl. Path., *29*, (1948): 141-148.

[2] Smith, L. DS., and Marsh, Hadleigh: The Immunization of Young Lambs Against Enterotoxemia. Am. J. Vet. Res., *14*, (1953): 408-410.

Transmissible Gastroenteritis in Pigs

KYU M. LEE, M.D., Ph.D.; MANUEL MORO, D.V.M., M.S.;
JAMES A. BAKER, D.V.M., Ph.D.

Ithaca, New York

IN RECENT years, reports have been made from various parts of the United States concerning serious outbreaks of transmissible gastroenteritis in swine. Doyle and Hutchings [1] in Indiana originally described and named the disease. These workers demonstrated that it was caused by a virus that produced a high mortality in baby pigs under 2 weeks of age. In 1947, Hutchings [2] reported the results of further work with gastroenteritis and confirmed his original observations concerning the serious nature of the infection. Feenstra, Thorp, Gray, and McMillen [3] found the disease prevalent in Michigan. Since then, it has become common knowledge that the disease occurs wherever swine are raised.

Field observations were made by Bay [4] in 1952, that showed newly farrowed pigs readily became infected, even in herds in which the disease had apparently subsided for several weeks. He also noted that if breeding were interrupted so that no pigs were born for three months, pigs born thereafter might remain uninfected, and this was recommended as a practical procedure to be followed in swine production. The appearance of a similar clinical syndrome in pigs of several herds in New York permitted isolation of virus strains and an investigation aimed at finding the means of maintenance and spread of the virus within a swine herd was initiated.

MATERIALS AND METHODS

Strains Used for Study.—In the spring of 1950, there occurred in a herd of swine at Saranac Lake, N. Y., a disease characterized by sudden onset, vomiting, profuse diarrhea, and rapid loss of weight. Nearly all of the animals in this herd were affected. The mortality was high in young pigs while older ones recovered after three to five days of illness. From a sick pig, portions of the small intestine were removed after autopsy, placed in vials, quickly frozen, and stored under dry ice refrigeration. Later, this material was thawed, made into a 10 per cent suspension in buffered saline with a glass grinder and other pigs were each fed 1 ml. of this material. When they became ill, material was obtained and stored for further study. The infective agent in this material was designated "New York I." An additional outbreak of gastroenteritis was observed in 1951, in the vicinity of Ithaca, N. Y. Again, material was obtained from a sick pig and the disease was transmitted successfully to other pigs. The infective agent in this material was designated "New York II."

In studies on filterability of the agent, suspensions were prepared as above from strain New York I and from New York II, centrifuged at 3,500 r.p.m. for ten minutes, and the supernatant fluid was filtered through a Seitz "EK" disc under 5 lb. pressure per square inch. In a test of infectivity, a pig was fed 1 ml. of a filtrate. Each filtrate produced symptoms of gastroenteritis. In further tests, intestinal mucosa was removed from these pigs, filtered and tested in a similar manner. The procedure was repeated a third time. Both strains were carried through three successive filtrations and, in every test, the filtrates produced illness.

Filtrates of intestine and portions of kidney, liver, lung, spleen, and brain that were shown to contain the infective agent were placed in the following mediums: 10 per cent horse serum broth, Schüffner's medium, PPLO medium, thioglycolate broth, and on blood-agar slants. Cultures were placed at 37 C. both under aerobic and anaerobic conditions and incubated for intervals as long as two weeks. In addition, blood was taken daily from infected pigs during the acute stage of illness and placed on blood agar plates. All cultures failed to show growth of organisms. In addition, a search for Salmonella and Shigella organisms was made on selective mediums. Feces or portions of intestinal tract shown to contain infective agent were cultured in selenite F medium for forty-eight hours and then placed on desoxycholate, on eosin methyl-

TABLE 1—Results of Cross-Immunity Studies on Recovered Pigs Showing Similarity Between New York Strains of Transmissible Gastroenteritis Virus and the Strain Obtained from Purdue University

	Test strain*		
Immunizing strain	N.Y. I	N.Y. II	Purdue
N.Y. I	0/3	0/2	0/5
N.Y. II	0/3	0/3	0/3
Purdue	0/4	0/2	0/4

* Denominator indicates number of animals tested, and numerator indicates those that showed signs of illness when given test virus.

From the Veterinary Virus Research Institute, New York State Veterinary College, Cornell University, Ithaca.

This work was aided by grants from Pitman-Moore Company, Armour and Company, and Fort Dodge Laboratories, Inc.

ene blue, on bismuth sulfate, and on SS agar plates. Neither Salmonella nor Shigella organisms were found in this material.

Blood taken from the pigs when they were moribund and portions of feces obtained from the intestinal tract of pigs affected by the disease were placed in lactose broth and then cultured on blood agar and MacConkey's agar plates. From these plates, ten isolations of bacteria of the coliform type were made and subcultured on horse-infusion agar plates at 37 C. for eighteen to twenty hours. After incubation, the organisms were taken up in sterile skimmilk and then divided into two portions. One portion was fed to pigs while the other was used to count the number of organisms that were being fed. Ten pigs ranging in age from 2 to 12 days were used in these tests, and after the count of organisms was made, each pig was found to receive from 300 million to 500 million organisms. None subsequently became ill.

TABLE 2—Production of Diseases in Pigs with Transmissible Gastroenteritis Virus

Route of inoculation	Results*	
	Elevated temperature**	Diarrhea and other signs
Oral	75/118	118/118
Parenteral	13/15	0/15
Contact	11/15	15/15

* Denominator indicates the number of animals inoculated, and the numerator indicates the number of animals showing reaction.

** Elevated temperature interpreted as readings above 39.5 C.

A strain of transmissible gastroenteritis virus was obtained through the courtesy of Dr. Hutchings and Dr. Bay of Purdue University, and designated by us as "Purdue strain." Using this strain and the two agents from New York pigs, comparisons were made by a reciprocal cross-immunity test. In the test, a group of 10 pigs was infected with New York I, 8 with New York II, and 12 with Purdue strain. After recovery, each group was divided into three lots and each lot was reinoculated either with the same strain or one of the others. Control pigs were included each time in a test for infectivity of test material. In all instances, pigs reacted to initial inoculation but, as can be seen in table 1, they did not react when test virus was given, indicating a similarity between the two New York strains and between them and the strain obtained from Purdue University.

From these studies, it was concluded that the agents designated New York I and New York II were capable of passing filters that retained bacteria and, since no bacterial organisms could be incriminated, it appeared that a filterable virus was the cause of the disease in the New York pigs. Furthermore, cross-immunity studies in pigs identified it with a strain of transmissible gastroenteritis virus obtained from the original workers on this disease.

Production of Disease.—All of the pigs used in these studies came from a herd maintained by the Institute under conditions designed to keep them free from infection. Over the years that this herd has been under observation, no evidence of enteric disease has been seen. In most instances, litters of pigs used for the study were removed from their mothers when they were 2 to 4 days of age and placed in an isolation unit where they would not be exposed to any unplanned infection. They were fed a milk substitute mixed with evaporated milk and a small amount of ferrous sulfate. A special cage was designed in order to allow the pigs to eat at any time. Older pigs were removed from the herd and held under observation in isolation units for several days before use.

After observation periods ranging from two to eleven days, pigs were inoculated by various methods: *(a)* each of 111 pigs varying in age from 2 to 120 days were fed 1 ml. of filtered or unfiltered intestinal mucosa from an infected pig, and 7 other pigs were inoculated in a similar manner with 1 ml. of blood from an infected pig; *(b)* 15 pigs were inoculated parenterally, each with 1 ml. of filtrate from intestinal mucosa from an infected pig; 6, when 6 days of age subcutaneously; 4, 19 days of age intramuscularly and 3, 19 days of age intraperitoneally. In addition, 2 pigs 6 days of age were given subcutaneously 1 ml. of unfiltered kidney suspension; *(c)* 15 pigs, 5 days of age, were placed in contact with other pigs that had been made ill. All pigs were observed at least once each day, rectal temperatures taken, and signs of illness were recorded in a uniform manner. In some animals, total white blood cell counts were made. Serums were collected from some of the pigs before inoculation and after recovery. In addition to these observations, some of the pigs were weighed to determine effects of the virus during acute illness and for long range effects. Also, from infected pigs at various intervals of time, specimens were taken for tests on presence, persistence, and concentration of virus. The results of infectivity tests are presented in table 2.

RESULTS

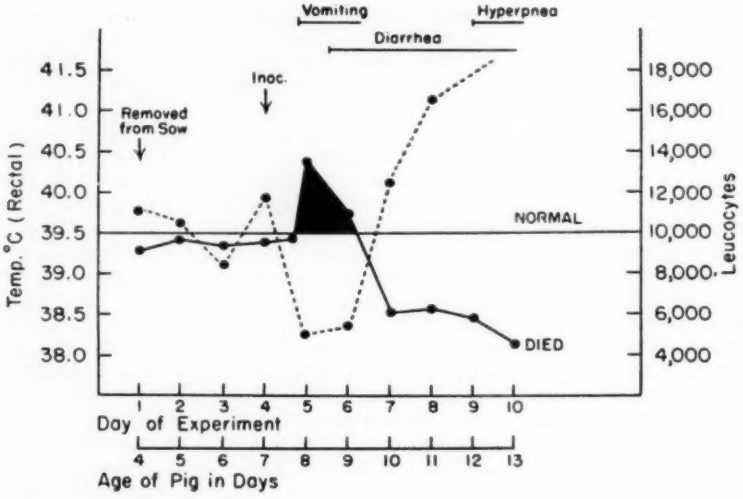
As shown in table 2, all animals developed diarrhea and other signs of illness following oral administration of infective ma-

TABLE 3—Mortality Produced in Pigs of Various Ages by Feeding Suspensions of Intestinal Mucosa from Infected Pigs

Exposure age (days)	Incubation period (hours)	Results*		
		Vomiting	Diarrhea	Mortality
1-5	12-18	+	+	21/21(100%)
6-10	18-36	+	+	31/46(67%)
11-15	24-48	+	+	6/20(30%)
16-120	24-72	—	+	1/24(4%)

* Denominator indicates number of animals tested and numerator indicates those that died.

terial. All 4 pigs that were inoculated intramuscularly, 3 of 6 pigs inoculated subcutaneously, and all 3 inoculated intraperitoneally showed a slight elevation of temperature as the only sign of infection. When the same material that had been used for the parenteral inoculation was fed to the pigs inoculated intramuscularly twelve days after the initial inoculation, they showed no signs of illness. The pigs inoculated subcutaneously were fed seven days afterward, and they became ill with characteristic signs of illness. All 15 pigs exposed to others that had been infected developed typical disease.



Graph 1 — Signs of illness shown by a pig infected with virus of transmissible gastroenteritis. (Solid line indicates the pig's temperature, the dotted line the leukocyte count.)

FEATURES OF DISEASE

Clinical.—Usually within twenty-four to forty-eight hours following inoculation, most pigs less than 15 days of age showed loose stools and elevated temperatures. The incubation period was as short as twelve hours in younger pigs and in older pigs was as long as ninety-six hours. Vomiting was observed in most cases but was less frequent in older animals. The most constant sign of illness was diarrhea characterized by frequent and violent discharge of yellowish or greenish feces that became watery in severely affected pigs. The leukocyte count usually was within normal limits for most pigs but, in some, a leukopenia of moderate degree developed during the febrile period. During recovery from illness, there tended to be leukocytosis. A typical case is shown in graph 1.

Of 59 cases in which death occurred, the interval from inoculation until death varied from three to seven days, with the exception of 2 pigs in which the period of time was nine and twelve days. In the 52 cases in which the animals recovered, the interval from inoculation until recovery averaged five and one-half days and ranged from one to twelve days. In young animals, the mortality was high and was 100 per cent in pigs inoculated under 5 days of age, 67 per cent in pigs between 6 to 10 days of age, and 30 per cent in pigs between 11 and 15 days of age. Pigs older than 16 days usually recovered with the exception of 1 pig, inoculated when 17 days old, that died. Data, obtained from the 111 cases and summarized in table 3, clearly show the relationship between the age of the animal and the severity of illness in that there was a decreased mortality with advancing age.

In cases with fatal termination, signs of dehydration and increased rate of respiration appeared rapidly. Just prior to death, pigs showed a dry skin, sunken eyeballs, and sometimes incoordination. The rectal temperature at the final stage was below normal. Animals became prostrated, went into coma, and death ensued.

Pathological.—The material available for gross and histological studies was provided by 77 pigs; 59 under 3 weeks of age, 18 that were 6 to 12 weeks of age. Twenty-one died from the infection; 56 were killed at various times after inoculation. To these were added 10 normal pigs, 4 days to 12 weeks of

age. Immediately after autopsy, the tissues were fixed in 10 per cent aqueous or alcoholic formalin solution and in a few instances in Zenker's solution. All sections were stained with hematoxylin-eosin and, in addition, those fixed in Zenker's solution by Wilhite's method.

Autopsy showed few changes grossly and these were mainly confined to the intestines. Both large and small intestines were always distended with gas and contained yellowish or greenish fluid. All other organs appeared normal except the mesenteric blood vessels were dilated and the kidneys appeared reddened in the medullary portion. In some cases, deposits of material were found in the renal pelvis. Cystic kidneys were seen in 3 pigs. In a few pigs, hemorrhages were observed in the stomach.

Histological examination of pigs sacrificed early in the disease showed a few dilated tubules in the kidneys that contained hyaline casts. Also, scattered degenerative changes in the cells of the tubules were seen. In pigs dying after prolonged illness, the tubules of the kidneys were dilated and filled with hyaline or granular casts. Lining cells of tubules were flattened, indicating pressure in the tubules (fig. 1 and 2). Some tubules were filled with leukocytic casts. Degenerative changes occasionally were observed in the nuclei of cells of convoluted tubules. The blood vessels of the medullary portion of the kidney were markedly congested and, in 1 case, hemorrhage was seen. Other organs presented only minor changes. In the intestine, the epithelial cells appeared normal and little, if any, cellular infiltration was seen. In 4 pigs, the cytoplasm of cells around the central vein of the liver appeared granular and translucent. These animals had suffered prolonged illness. Hyperpigmentation and shrinkage of large motor cells in the cerebellum and cerebrum were observed in a few pigs. Microscopic examinations of the heart muscle, adrenal gland, lung, bladder, and meninges of pigs showed no significant changes. Extensive search revealed no inclusion bodies in tissues. No lesions were found in 14 pigs that were sacrificed four to eleven weeks after inoculation. Examination of uninoculated pigs showed no changes.

Serological. — Cross-immunity studies were reported above for strain identification and, in making these comparisons, it was demonstrated that pigs after recovery showed no signs of illness when reinoculated with infective material. Also, in studies of natural disease, it had been noted that pigs from a sow were immune if the sow had been exposed previously. These immunity phenomena prompted efforts to demonstrate antibody.

In a demonstration of antibodies, serums were obtained from pigs that had recovered from the disease and neutralization of the virus attempted. In one test, undiluted serum was mixed with an equal portion of a 10^{-2} dilution of a suspension of intestinal mucosa from an infected pig. In like manner, serum from a pig before infection was mixed with a hundredfold less virus or a 10^{-4} dilution of mucosal suspension. The mixtures were incubated for two hours at 37 C. and 2 ml. of mixture was fed to each of 2 pigs that were 10 days of age. These tests were repeated with serums from recovered pigs with dilution of virus increased to 10^{-3} in the second test and 10^{-4} in the third. In each of these tests, serums from uninoculated pigs were included with virus correspondingly diluted a hundredfold. All pigs developed characteristic signs of illness and it was concluded that neutralization was not effected.

In a serum protection test, serums were obtained from 2 pigs after recovery from illness and pooled. To a group of 2 pigs that were 6 days of age, each was given 10 ml. of the pooled serums orally and 10 ml. intramuscularly. After one day, each pig was given orally 1 ml. of a 10^{-1} suspension of intestinal mucosa from an infected pig. A similar group of litter mates was given virus only. This test was repeated in pigs 3 days of age but the amount of virus was reduced to a dilution of 10^{-3}. All pigs died, although those given serum lived slightly longer.

Epidemiological.—In a study to determine whether transmission would occur through contact exposure, 2 pigs were placed in the same cage and 1 was given orally 1 ml. of intestinal suspension from an infected pig while the other was left uninoculated. The inoculated animal developed illness as usual. The exposed animal developed characteristic signs of illness twelve to twenty-four hours after the inoculated animal showed signs of illness. This experiment was repeated and similar results were obtained.

In another experiment designed to demonstrate whether virus would spread by routes other than by cage contact, 2 pigs

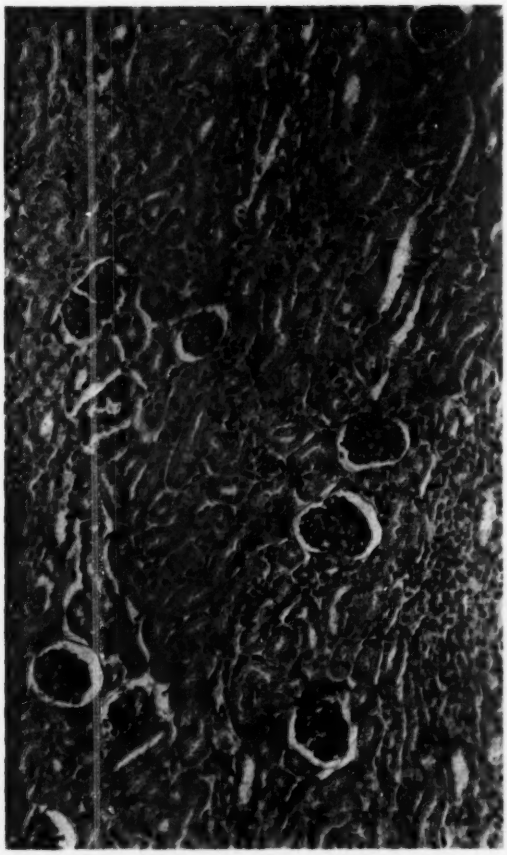


Fig. 1—Histological section of kidney from an uninoculated pig. Specimen taken when pig was 4 days old. Fixed in alcohol formalin. Stained with hematoxylin-eosin. x 210.

were placed in a cage and, in this same room but in a separate cage, 2 other pigs were placed. Utmost care was taken to avoid mechanical transfer of any material that might contain virus from one group of pigs to the other. To one of the groups 1 ml. of infective material was given each orally. They became ill in the usual manner. The exposed pigs showed characteristic signs of illness twelve to twenty-four hours later. This experiment was repeated twice with the same results and indicated that the disease spread by routes other than direct contact.

STUDIES ON THE VIRUS

In the course of studies on the disease pattern in pigs, it seemed worthwhile to make measurements on the concentration of the virus in various organs, its ability to persist in various organs, and the relationship of persistence of virus to weight gains.

Concentration of Virus in Various Organs.—From a pig that had been inoculated orally with 1 ml. of a suspension of intestinal mucosa from an infected pig, blood was obtained two days after inoculation and tenfold dilutions to make 10^{-1} and 10^{-2} were prepared in buffered saline. From the same pig, a 10 per cent suspension in saline of intestine, of kidney, of liver, of lung, of spleen, and of brain also were made and, after centrifugation at 1,500 r.p.m. for five minutes, dilutions of the supernatant fluid of each were prepared in a manner similar to that for blood but ranged to higher dilutions. Seven litters of pigs, 6 to 9 days of age, were divided into groups of 7 animals

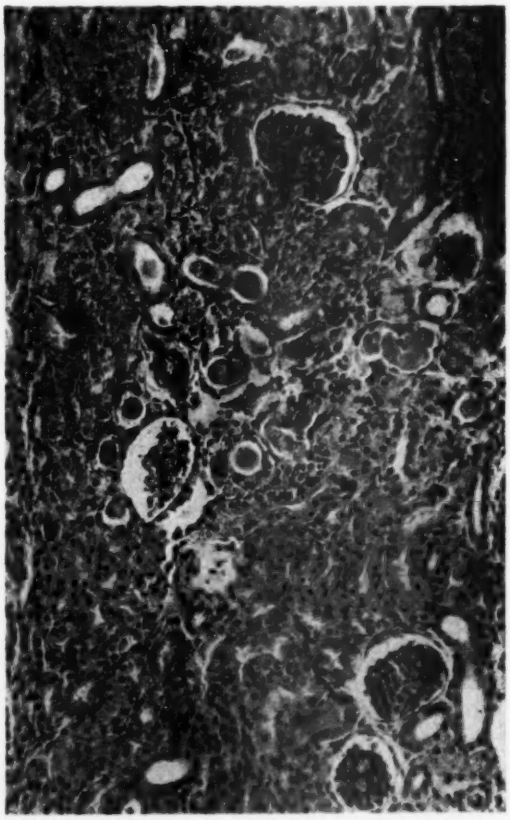


Fig. 2—Histological section of kidney from a pig inoculated with transmissible gastroenteritis virus. Specimen taken seven days after inoculation when pig was 19 days old. Note dilation of tubules and casts. Fixed in alcohol formalin. Stained with hematoxylin-eosin. x 210.

with each litter represented in a group. To one group, each of the various dilutions of blood were fed in a 1-ml. amount to a pig. In a similar manner, dilutions of the organ suspensions were tested one to each group so that it was possible to determine the relative concentration of virus. Using another infected pig, these tests were repeated and the findings are presented in table 4.

As can be seen in table 4, virus was present in a concentration of 1×10^1 infective doses per milliliter in the blood. A concentration of 1×10^6 infective doses per gram of tissue was found in the intestine, 1×10^6 for the kidney, 1×10^3 for the liver, 1×10^4 for the lung, and 1×10^1 for the spleen and brain.

Persistence of Virus in Various Organs.—The presence of virus in certain organs and excretions from 13 pigs that had recovered from infection was determined at various intervals of time. Since the signs of illness were referable to the intestinal tract, feces was studied principally although blood and urine were studied also. In addition, several pigs were killed and tests for virus made in kidneys, in lungs, and in intestines. In collection of urine, blood, and feces, specimens were obtained before inoculation and thereafter at weekly intervals throughout the duration of study that lasted from nine to eleven weeks. At this time, the pigs were necropsied and tests for virus made of their intestines, lungs, and kidneys. Immediately after collection, all specimens were frozen and held under dry ice refrigeration until tested.

In testing for virus, each specimen was thawed and prepared for inoculation. In a test of feces, 10 ml. of supernatant fluid from a 20 per cent suspension in buffered saline that had been centrifuged at 3,200 r.p.m. for ten minutes was fed. In a test of urine, 10 ml. of undiluted urine was given orally. In a test of blood, 4 to 5 ml. of a 50 per cent suspension in buffered saline was fed. For a test of intestines, of lungs, or of kidneys, portions of each were made into a 20 per cent suspension in saline and 5 ml. fed to each test pig. The test pigs whose ages varied from 4 to 15 days came from the laboratory herd and, in the test, were kept isolated individually in order to avoid cross infection. The presence of virus was determined by clinical signs of illness and by pathological lesions. Pigs that showed no evidence of infection were later given infective virus, and if they became ill, the test specimen was considered free from virus.

TABLE 4—Concentration of Transmissible Gastroenteritis Virus in Various Organs of Infected Pigs

	Results*						
Dilution	Blood	Intestine	Kidney	Liver	Lung	Spleen	Brain
10^{-1}	2/2	2/2	2/2	NT	NT	2/2	2/2
10^{-2}	0/2	2/2	2/2	2/2	NT	0/2	0/2
10^{-3}	NT	2/2	2/2	2/2	2/2	0/2	0/2
10^{-4}	NT	2/2	2/2	0/2	2/2	NT	NT
10^{-5}	NT	2/2	2/2	0/2	0/2	NT	NT
10^{-6}	NT	2/2	2/2	NT	NT	NT	NT
10^{-7}	NT	0/2	0/2	NT	NT	NT	NT

* Denominator indicates number of animals tested. Numerator indicates those showing signs of illness. NT indicates not tested.

TABLE 5—Elimination of Virus in Feces in Relation to Time

		Accumulation totals		
No. weeks after inoculation	No. pigs shedding virus*	No. pos.	No. neg.	Percentage showing virus
2	1	13	0	100
3	3	12	1	92
4	3	9	4	68
5	4	6	7	46
6	1	2	11	16
7	0	1	12	8
8	1	1	12	8
9	0	0	13	0

* These pigs shed virus for the length of time indicated in first column but failed to eliminate virus beyond that period.

Tests from 1 pig, one week after inoculation, showed virus in the lungs and in the intestines. From 2 pigs tested two weeks after inoculation, virus was present in the lungs and intestines of both and in the kidneys of 1. Subsequent tests showed virus in the intestines of the 1 pig killed four weeks after inoculation but not in the kidneys. In later studies made on 13 pigs nine to eleven weeks after inoculation, no virus was found in any organ.

Blood was tested from 9 pigs one week after inoculation and 3 showed virus, but no virus was found in 10 pigs tested two weeks after inoculation or in 1 pig tested four weeks afterward.

Virus was not found in urine from a pig one week after inoculation although it showed virus in the blood. Although virus was found to be present in the kidneys of 1 of the 2 pigs whose kidneys were studied two weeks after inoculation, there was no virus found in the urine of either pig.

Since virus appeared to persist longer in the intestines than in other organs, more extensive tests were made of the fecal specimens that had been obtained. Since a possibility existed that virus was eliminated in the feces intermittently, 10 pigs that showed virus in a test and showed no virus the next week were retested again on the following week. In this test, none showed virus. Conversely, from 10 pigs whose feces showed virus in a test, the fecal specimens obtained the preceding week were tested. All showed virus. It would seem that virus elimination was not intermittent. Actual tests, therefore, consisted of finding for each pig the longest interval virus was present in feces. Accordingly, specimens obtained at the longest interval after inoculation were tested and, if no virus was found, specimens obtained at shorter time intervals were tested until virus was demonstrated. As shown in table 5 for 13 pigs, virus persisted for two weeks in 1 pig, three weeks in 3, four weeks in 3, five weeks in 4, six weeks in 1, and eight weeks in 1.

It thus appears that during acute illness, virus was present in the blood, lungs, and kidneys but soon after recovery was no longer present. Interestingly enough, it was never found in the urine, although it was present in the kidneys. Virus persisted in the intestinal tract, however, and was eliminated in the feces for variable periods of time in different pigs. These results in table 5 are expressed as a calculated percentage of pigs tested that showed virus in relation to time of virus persistence and varied from none at nine weeks to 100 per cent at two weeks after inoculation with a 50 per cent persistence between four and five weeks after inoculation.

Effect on Weight.—In the course of these studies, it was noted that some infected pigs after apparent recovery grew slowly, although they showed no other detectable signs of illness. This alteration of growth was more evident in pigs that showed persistence of virus in feces than in those free of virus. It seemed worthwhile to define more precisely this effect of the disease on weight. Accordingly, a litter of 8 pigs was obtained when they were 4 days old and 7 of them placed in one isolation room while the other pig, to be kept as the uninoculated control, was placed in a separate room. Weighing of all pigs began two days later and was continued daily thereafter. When the pigs were 10 days old, each animal in the group of 7 pigs was given virus in the usual manner by feeding with 1 ml. of a suspension of intestinal mucosa from a pig infected with transmissible gastroenteritis virus. At the time of inoculation and afterward, feces were obtained at weekly intervals and tested for virus. In addition, tests were made of feces from the uninoculated pig. This experiment was repeated with another litter of 6 pigs, 5 of which were inoculated while 1 was kept uninoculated.

Of the 12 inoculated pigs in the two litters, 8 died and 4 survived, 2 in each litter. In the first group of pigs, the 2 survivors showed persistence of virus in the feces for thirty-five days and, in the other group, 1 pig showed no virus in the feces when tested after twenty-one days while the other showed virus for forty-two days. The uninoculated pigs showed no signs of illness and, in tests of feces, no virus was found.

The effect of virus on weight is shown in graph 2 as average weights of 2 inoculated pigs that showed persistence of virus for thirty-five days, average weights of the 2 controls, and the weight of the pig in which virus did not persist. It can be seen that the pigs gained at least 100 Gm. daily up to the time of inoculation. Afterward, the inoculated pigs started to lose weight while the control pigs continued to gain. After apparent recovery from the disease, the inoculated pigs gained less weight than the controls and, of the 3 inoculated pigs, the 2 that eliminated virus for thirty-five days gained more slowly than the 1 that eliminated no virus after twenty-one days. At the age of 80 days when the study was terminated, the controls had an average weight of 34.8 kg. (76.5 lb.), the 2 pigs that showed persistence of virus had an average weight of 18.4 kg. (40.5 lb.), and the pig that did not have virus persisting in the feces had a weight of 29 kg. (63.8 lb.). The inoculated pig not placed in the figure that showed virus in the feces forty-two days after inoculation weighed 20.9 kg. (46 lb.).

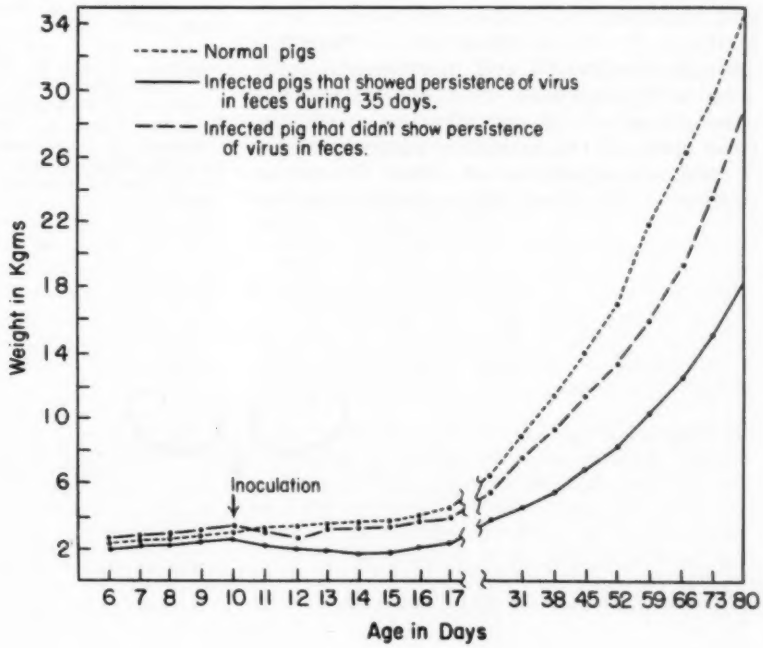
DISCUSSION

In studies on a disease occurring in pigs in New York State characterized by profuse diarrhea, vomiting, rapid loss of body weight, and high mortality, two strains of an infective agent were transmitted successfully to other pigs. The agents were found capable of passing through filters that retained ordinary bacteria. No bacteria were revealed either microscopically or in cul-

tures of various organs or of filtrates of the intestines that were known to be infective. Since the disease could be transferred serially in pigs after filtration, it appeared toxins were eliminated. These findings suggested that the disease was caused by a filterable virus. Clinical features of illness shown by naturally infected pigs and in experimental animals were similar in every respect to the clinical syndrome described by Doyle and Hutchings [1] and later by others. In their studies, Doyle and Hutchings named the disease transmissible gastroenteritis and concluded that the cause was a virus. A strain of this virus was obtained from Drs. Hutchings and Bay at Purdue University and, in cross-protection tests, it was found to be closely related, if not identical, with the strains obtained from the New York pigs. Therefore, resemblance of clinical features, similarity of properties of the etiological agent, and immunological relationship make possible the conclusion that the disease in the New York pigs was transmissible gastroenteritis.

The disease was readily produced by the oral administration of infective material but inoculations made subcutaneously, intramuscularly, and intraperitoneally did not produce typical disease. As shown by measurements of virus from various organs, both kidney tissue and intestinal mucosa contained virus in a higher concentration than blood, liver, spleen, or brain.

In attempts to explain spread and maintenance of the virus, studies were directed toward persistence of virus within the body of the natural host. Infective virus was found in the kidneys of 1 pig in the acute stage of illness and in the blood of another, but neither pig showed virus in the urine. Also, tests for the presence of virus in the kidneys of 14 pigs after the acute phase had subsided gave uniformly negative results. In the light of these observations it appeared that unlike the virus of infectious hepatitis in dogs,[5] the virus of transmissible gastroenteritis in pigs does not persist in the kidneys. Also failure to persist in the blood showed it unlike lymphocytic choriomeningitis in mice.[6] In the tests for virus in the lungs, it was obtained from 2 pigs shortly after inoculation but tests of lungs from 13 pigs several weeks after inoculation showed no virus. Unlike psittacosis virus which persists in the respiratory tract of human beings,[7] this virus did not remain in the lungs. However, like poliomyelitis virus in human beings,[8] transmissible gastroenteritis virus was found in feces for as long as eight weeks after inoculation. It appears that the disease primarily affects the intestinal tract although the kidneys show lesions and for a time contain virus in a high



Graph 2 — Effect of transmissible gastroenteritis virus on weight of pigs.

concentration. Since virus was shown to persist in the intestinal tract and to be eliminated in the feces and since infection was produced by the oral route, this method may account for the spread of disease within a herd. Furthermore, this finding conforms with the observation of Bay [4] that interruption of farrowing for 4 to 8 weeks will free a herd of infection.

During the acute stage of illness, all infected pigs lost weight rapidly, probably as a result of dehydration and inappetence. After the acute phase of illness had ended, surviving pigs were stunted and gained weight slowly. In tests where weights were recorded after apparent recovery, a significant difference was found between uninfected controls and infected pigs that showed persistence of the virus in the intestines. Also, inoculated pigs in which virus persisted gained less weight than those in which virus did not persist. Thus, a theory might be advanced that the difference in weights of many pigs under field conditions is caused by freedom from virus infection or, after infection, ability of the virus to persist and produce stunting of growth.

SUMMARY

From an enteric disorder of pigs in New York State, a virus was procured that produced signs of illness resembling the naturally occurring disease. Field observations and experimental studies indicated a similarity to the clinical syndrome of transmissible gastroenteritis and in cross-immunity studies it appeared that the virus was closely related, if not identical, with the type virus of transmissible gastroenteritis.

Oral administration of either filtered or unfiltered intestinal suspensions from infected animals readily and regularly produced characteristic signs of illness in pigs and, since parenteral inoculation of virus did not, it appeared that the natural route of infection was by way of the mouth. In the acute phase of illness, virus was found in the blood, liver, spleen, brain, and lung in a low concentration but was present in high concentration in the intestine and kidney. Virus was recovered from feces of infected pigs for intervals of time extending two to eight weeks after inoculation. Since virus was recovered from the lungs and kidneys of infected pigs for only a short time after inoculation, from the blood during the period of clinical illness, and in no instance from the urine, it would appear that persistence of virus in the feces has epidemiological significance. Infected pigs that showed persistence of virus in the intestines were stunted and did not gain weight as well as pigs that did not harbor virus, or uninfected pigs.

References

[1] Doyle, L. P., and Hutchings, L. M.: A Transmissible Gastroenteritis in Pigs. J.A.V.M.A., *108*, (1946): 257.

[2] Hutchings, L. M.: Gastroenteritis in Young Pigs. Vet. Med., *42*, (1947): 297.

[3] Feenstra, E. S., Thorp, F., Gray, M. L., and McMillen, W. N.: Transmissible Gastroenteritis of Baby Pigs. J.A.V.M.A., *113*, (1948): 573.

[4] Bay, W. W.: Transmissible Gastroenteritis in Swine. Field Herd Studies. J.A.V.M.A., *120*, (1952): 283.

[5] Poppensiek, G. C., and Baker, J. A.: Persistence of Virus in Urine as Factor in Spread of Infectious Hepatitis in Dogs. Proc. Soc. Exptl. Biol. and Med., *77*, (1951): 279.

[6] Olitsky, P. K., and Casals, J.: Viral and Rickettsial Infections. Edited by T. M. Rivers. J. B. Lippincott Co., Philadelphia, (1952): 247.

[7] Meyer, K. F., and Eddie, B.: Human Carrier of the Psittacosis Virus. J. Infect. Dis., *88*, (1951): 109.

[8] Brown, G. C., Francis, T., Jr., and Ainslie, T.: Studies of the Distribution of Poliomyelitis Virus. V. The Virus in Familial Associates of Cases. J. Exptl. Med., *87*, (1948): 21.

Experimental Tocopherol Deficiency in Young Calves

J. W. SAFFORD, D.V.M.; K. F. SWINGLE, Ph.D.; H. MARSH, D.V.M.

Bozeman, Montana

IT HAS BEEN demonstrated that muscular dystrophy can be produced in several species of animals by feeding a vitamin E-deficient diet. Information is lacking on the effects of a vitamin E-deficient diet on calves. To obtain this information and as an approach to the "white muscle" disease problem in calves, an attempt was made to determine the effects of a tocopherol deficiency on newborn calves.

LITERATURE CITED

Vawter and Records [1] called attention to the similarity of symptoms and anatomical changes occurring in spontaneous muscular dystrophy of calves to those induced in lambs, rats, rabbits, guinea pigs, and some birds by diets deficient in vitamin E. The lesions observed in beef calves in western Montana [2] were similar to those described in rabbits and guinea pigs by Goettsch and Pappenheimer;[3] to those in rats as described by Papenheimer;[4] Evans, Emerson, and Telford;[5] Follis;[6] to those in mice as described by Pappenheimer;[7] to those in lambs as described by Willman, Asdell, Grams, and Hagan;[8] Marsh;[9] Culik, Bacigalupo, Thorp, Luecke, and Nelson;[10] and to those in calves as described by Vawter and Records.[1]

Goettsch and Pappenheimer [3] demonstrated that vitamin E deficiency produced muscular dystrophy in guinea pigs and rabbits, and Pappenheimer [7] likewise experimentally produced muscular dystrophy in mice. Shimotori, Emerson, and Evans [11] demonstrated that muscular dystrophy in guinea pigs could be prevented with vitamin E while Goettsch and Pappenheimer [12] demonstrated that *alpha*-tocopherol prevented it in rats. Adamstone, Frider, and James [13] produced muscular degeneration in pigs on a vitamin E-deficient ration. Whiting, Willman, and Loosli [14] attributed the cause of muscular dystrophy in lambs to avitaminosis E. Willman, Loosli, Asdell, Morrison, and Olafson [15] and Marsh [16] demonstrated that muscular dystrophy in young lambs could be prevented and cured with administrations of vitamin E. Culik *et al.*[10] experimentally produced muscular dystrophy in young lambs by feeding a synthetic milk deficient in vitamin E.

Gullickson, Palmer, Boyd, Nelson, Olson, Calverly, and Boyer [17] reported they were able to raise successive generations of calves from cattle on rations low in vitamin E. Their reports show that there were 12 deaths in the original 30 experimental cattle between the ages of 21 months and 5 years. No gross lesions were observed and the cause of death was undetermined. Gullickson and Calverly [18] reported the cause of deaths to be cardiac failure in the adult cattle on the rations low in vitamin E. Muscular dystrophic lesions were not reported.

Latschar, Wise, Parrish, and Hughes [19] found a lowering of the prepartum and postpartum serum tocopherols in dairy cattle. Parrish, Wise, and Hughes [20] demonstrated the rapid decline in colostrum tocopherols in the early milks of cattle. Parrish, Wise, Latschar, and Hughes [21] were able to show considerable differences between the serum tocopherol levels of the dam and of the newborn calf.

During the time the experimental feeding was done on the calves reported in this paper, the findings of Blaxter, Watts, and Wood,[22] Blaxter and Wood,[23] and MacDonald, Blaxter, Watts, and Wood [24] were published. The results of their work demonstrated that muscular dystrophy could be produced in young calves maintained on a vitamin E-deficient diet.

EXPERIMENTAL PROCEDURE

Six newborn, male calves (Jerseys, D1, D2, C5, and C6; and Holstein-Friesians, D3 and D4) were placed on a tocopherol-deficient, synthetic milk diet following three feedings of their dam's colostrum. A gelatin capsule containing 74 mg. of *d-alpha*-tocopherol acetate in oil was given the control calves, C5 and C6, every twelve hours following each feeding throughout the experiment.

On the first day, each calf received 75,000 I. U. of vitamin A and 7,500 I. U. of vitamin D, which was followed by the daily administration *per os* of 5,000 I. U. of vitamin A and 500 I. U. of vitamin D in a gelatin capsule. A 250-mg. tablet of ascorbic acid was administered to each calf daily.

The synthetic milk fed during this experiment was similar in content and preparation to that described by Wiese, Johnson, Mitchell, and Nevans,[25] modified by Luecke, Culik, Thorp, Blakslee, and Nelson,[26] and further modified by Culik *et al.*[10]

All calves were fed at the rate of 1 lb. of synthetic milk to 10 lb. of live weight of calf per day, divided equally into two feedings. The calves

---

Paper 322, journal series, from the Montana Veterinary Research Laboratory (Montana Experiment Station and Livestock Sanitary Board cooperating), Agricultural Experiment Station, Montana State College, Bozeman.

The writers express their appreciation to the following: Montana State College Dairy Division for supplying the calves and for the assistance and equipment in preparing the synthetic milk; Harold W. Gregg, M.D., and M. A. Gold, M.D., Butte, Mont., for reviewing the electrocardiograms; Distillation Products, Inc., Rochester 13, N. Y., for supplying the molecular distilled lard.

were fed at 8:30 a.m. and 8:30 p.m. each day. The amount of milk to be fed at each feeding was warmed to 40 C. in a steaming oven just prior to feeding. The milk was fed in Coyner calf pails with rubber nipples. Following each feeding,

Fig. 1—Alpha-tocopherol-deficient calf D1, 29 days old. Unable to stand unassisted.

the pails were disassembled, washed, rinsed, and sterilized in an autoclave.

Each calf was assigned two pens. All pens were constructed to be separate and apart. A thick bedding of sawdust covered a cement floor in each pen. The calves were alternated from one pen to the other every three days. Following the removal of a calf from a pen, that pen was immediately cleaned, washed, and disinfected with a 2 per cent solution of sodium hydroxide, thoroughly rinsed, and allowed to dry.

The heart rates, obtained with a stethoscope applied to the chest wall, and the rectal temperatures were recorded twice daily for each calf at the times of feeding.

Electrocardiograms were obtained from each calf at weekly intervals. A Sanborn "cardiette," No. 202, was used with tracings recorded from each of three leads attached to the two front legs and the left rear leg.

Each calf was weighed once a week.

At weekly intervals, blood was taken from the jugular vein for erythrocyte, leukocyte, and differential cell counts, for hemoglobin and *alpha*-tocopherol determinations. Hemoglobin was determined photometrically by the acid-hematin method. The *alpha*-tocopherol determinations were made using methods described by Quaife and Harris.[28]

Urine samples were collected every three days for pH determinations, albumin, bile, diacetic acid, glucose, occult blood, acetone, specific gravity, creatine, and creatinine tests. The samples were collected in a Davol daytime female urinal similar to those used on sheep as described by Beeson, Pence, and Holm.[29] The pH was determined with the Fisher "alkacid" test ribbons. The presence of albumin was determined by the 20 per cent sulfosalicylic method. The Smith iodine reagent test was used to determine the presence of bile. Gerhardt's method was used to determine the presence of diacetic acid. Moore-Heller's method was used to test for the presence of glucose. The benzidine test was used to determine the presence of occult blood. The Jackson-Taylor modification of Legal's test was used to determine the presence of acetone. The creatine and creatinine determinations were made by the method of Folin.[30]

A sample of the colostrum fed to the newborn calf was taken for *alpha*-tocopherol determinations, which were made by methods described by Quaife.[31]

Tissue sections were stained with hematoxylin and eosin for histopathological study.

Following the development of muscular dystrophy symptoms, *alpha*-tocopherol acetate was administered subcutaneously to calf D2 as follows: 890 mg. on the forty-fifth day, 500 mg. on the forty-seventh day, 1,000 mg. on the forty-ninth and on the fiftieth days. It was administered to calf D4 as follows: 1,000 mg. on the forty-fourth day, 500 mg. on the forty-sixth day, and 1,000 mg. on the forty-eighth day.

RESULTS

Clinical Signs of Deficiency.—Calf D1.—Weakness of the leg muscles, first evident on the fourteenth day as a wobbly, staggering gait, became increasingly severe until on the twenty-seventh day the calf was

Fig. 2—Alpha-tocopherol-deficient calf D2 showing typical leg crossing.

able to stand about five minutes only before the muscles of the fore and hind limbs started trembling. This trembling developed quickly into such a violent shaking

Fig. 3—Alpha-tocopherol-deficient calf D2, 31 days of age, showing flexed carpus, relaxed rear fetlock, crossed hind legs.

that the calf was forced to lie down, whereupon the tremors ceased. On the thirtieth day the calf was unable to rise (fig. 1). In addition, there developed during this period a relaxation of the fetlocks, a slight toe spread, and an elevation of the suprascapular cartilages, becoming more marked as the disease progressed. The neck muscles were also affected so that on the thirty-third day the calf was unable to lift its head from the ground.

Involvement of the tongue was evidenced by a repeated extension and peculiar curling of the tongue on the twentieth day. Two days later there was considerable difficulty in cupping the tongue around the rubber nipple at feeding time, and by the twenty-seventh day this difficulty had become so severe that it required 20 minutes for the calf to consume 3½ lb. of milk.

On the thirty-third day the calf, unable to stand or even to raise its head, unable to consume its milk, and showing a subnormal temperature, was killed, and an autopsy was performed.

Calf D2.—Generally, development of the deficiency symptoms in this calf paralleled those in calf D1. The early evidence of leg weakness took the form of leg crossing (fig. 2) and hock interference, resulting in a wobbly-rolling-floppy movement, as contrasted with the staggering gait of calf D1. Extreme difficulty in getting to his feet was experienced from the twenty-ninth to the thirty-fifth days. Muscle tremors were pronounced when the calf did stand for a few minutes. From the thirty-seventh to the thirty-ninth days there was a temporary improvement, the calf being able to rise without much difficulty, but starting on the fortieth day a gradual exacerbation soon left the calf unable to stand.

A flexion of the carpal joints developed (fig. 3). The toe spread of the hind feet (fig. 4) was more marked than in calf D1 but the development of difficulty in feeding, while similar, came about more slowly.

To observe the effect of the administration of *a*-tocopherol, after severe muscular symptoms had developed, calf D2 was injected subcutaneously on the forty-fifth day with 890 mg. of *alpha*-tocopherol acetate. The following day there was a noticeable increase in general muscle tone, and the calf sucked the nipple with much more vigor. Since the leg weakness was less pronounced, and leg crossing and hock interference did not occur, a second dose (500 mg.) of *alpha*-tocopherol acetate was injected on the 47th day which was followed by further improvement. On the

Fig. 4—Alpha-tocopherol-deficient calf D2 showing typical toe spread.

forty-ninth day a dose of 1,000 mg. of the vitamin was injected, followed on the fiftieth day by a final dose of 1,000 mg. Improvement continued, the calf capered with vigor, and it had no further difficulty in nursing.

On the fifty-first day, pneumonia developed and the calf was killed for autopsy.

Calf D3.—The first sign of deficiency was observed on the seventeenth day, when the calf took twice as long as usual to consume its milk. However, on the following day the rate of feeding was again normal, and no further tongue symptoms were noted until the thirty-eighth day when the calf experienced difficulty in sucking the nipple and swallowing.

Weakness in the skeletal muscles was first observed on the twenty-fifth day when the suprascapular cartilages were extending 1¼ cm. above the spinous processes, and the scapulae were not held in the normal proximity to the chest. Finally, on the thirty-eighth day the cartilages were 6 cm. above the spinous processes. On the twenty-ninth day, when the calf was standing, the muscles of the legs showed a tremor which became a pronounced shaking within ten minutes. Two days later the calf was unable to rise unassisted and, when lifted to its feet, could stand only twenty or thirty seconds, on the tips of its toes with the carpal joints flexed, violently shaking, before it collapsed (fig. 5). The calf was at ease while lying, with its head up and ears erect (fig. 6). Its appetite was good and it consumed milk with vigor. On the thirty-third day the calf began to show a general improvement and could gain its feet with great effort and stand for about three minutes. On the thirty-fifth day, it was able to stand for twenty minutes. However, the improvement stopped and by the thirty-eighth day a general muscular weakness was again evident. The calf no longer attempted to rise and on the thirty-ninth day a pronounced toe spread was observed for the first time.

Fig. 5—Alpha-tocopherol-deficient calf D3 unable to stand on thirty-first day.

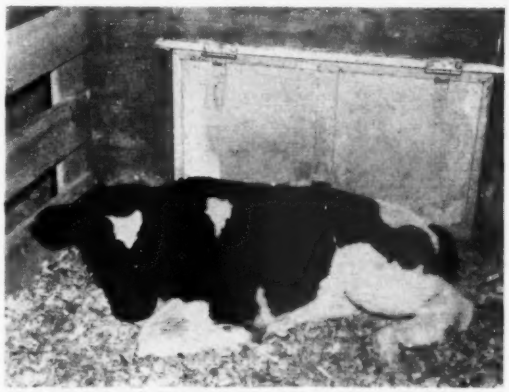


Fig. 6—Alpha-tocopherol-deficient calf D3 unable to stand. Normal appearance while lying.

On the fortieth day, pneumonia had developed and the calf was killed and autopsied.

Calf D4.—The first sign of deficiency was the extension, on the twenty-fourth day, of the suprascapular cartilages 2½ cm. above the spinous processes. From this time until the forty-third day developments were similar to those of calf D3. On the forty-third day, however, a neck weakness appeared, and the calf was unable to raise its head above the level of the long axis of the body (fig. 7). While lying down, its head rested on the ground. The scapulae were very prominently winged (fig. 7). The rotating, wobbly motion of the hind legs was similar to that of calf D2, while the forelegs had a quick, jerking, buckling action at the carpus and at the humeroradial and ulnar articulations.

This calf was used to observe the effects of tocopherol therapy initiated after the development of pronounced muscular symptoms. At 9 a.m. on the forty-fourth day, 1,000 mg. of *alpha*-tocopherol acetate was injected subcutaneously and by 8:30

p.m. the calf had recovered its ability to suck the nipple effectively, to swallow in a normal manner, to raise its head to shoulder level, and to move it laterally one fourth of the way back. Two days later, when it could raise its head and bend its neck in a normal manner, a second dose, 500 mg., of tocopherol was injected, with a third dose of 1,000 mg. on the forty-eighth day. Recovery appeared complete on the fiftieth day. The calf was destroyed and autopsied on the fifty-first day.

Control Calves C5 and C6.—Calves C5 and C6 remained vigorous and playful with good muscular action and control throughout the experiment. They were killed and autopsied on the forty-eighth and the forty-third days, respectively.

Other Clinical Signs of Deficiency.—Calf D1 had diarrhea on the eleventh, twelfth, and the twenty-first to twenty-third days.

Calf D2 had diarrhea on the tenth, the twenty-second to twenty-fourth, and the thirty-second to thirty-fourth days. Pneumonia was present in varying degrees from the twenty-first to fifty-first days.

Calf D3 had diarrhea on the third, fourth, eleventh, and thirty-second to thirty-fourth days. Pneumonia was apparent on the thirty-ninth day.

Calf D4 had diarrhea on the eighth, ninth, fifteenth, and sixteenth days.

Control calf C5 had diarrhea on the second, seventeenth to nineteenth, twenty-fifth, twenty-sixth, thirty-fifth, and thirty-sixth days.

Control calf C6 had diarrhea and pneumonia from the second to tenth days, twelfth, thirteenth, sixteenth, seventeenth, and thirty-fourth days.

Weight Gains.—Graph 1 illustrates the comparative weight gains of the calves on this experiment.

Temperatures.—The temperatures of all the calves remained within normal limits with the following exceptions: All showed rises from 103 to 105.3 F. coincidental with the occurrence of diarrhea and pneumonia. Calf D1 had a temperature of 98.5 F. just prior to autopsy on the thirty-third day.

Hematology.—The erythrocyte counts remained within normal limits. Leukocytosis occurred coincidental with the presence of diarrhea and pneumonia.

The differential leukocyte counts of both the deficient and control calves remained within normal limits, with the exception of fluctuations in both groups coincidental with the occurrence of diarrhea and pneumonia. All eosinophil, basophil, and monocyte counts were considered to be normal.

The hemoglobin determinations varied from 10.3 to 13.3 per cent on the control calves and from 9.6 to 15.9 per cent on the deficient calves.

Blood plasma *alpha*-tocopherol was not present in the calves on the deficient diet

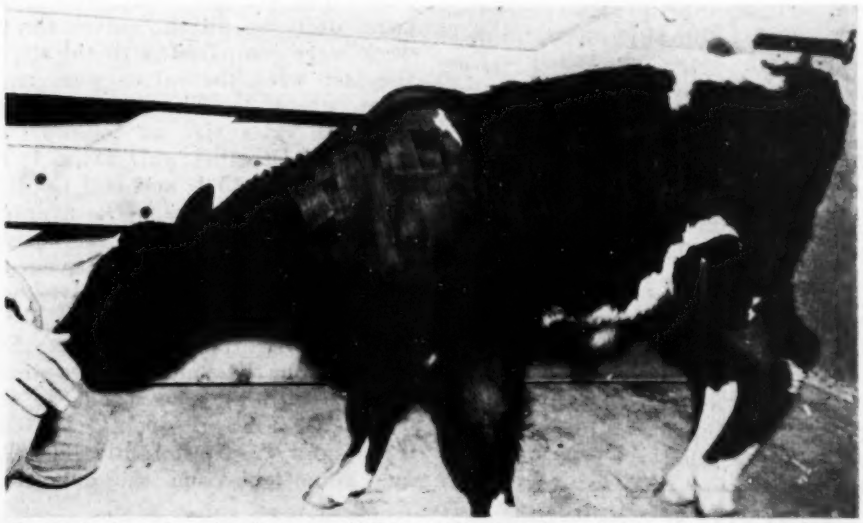
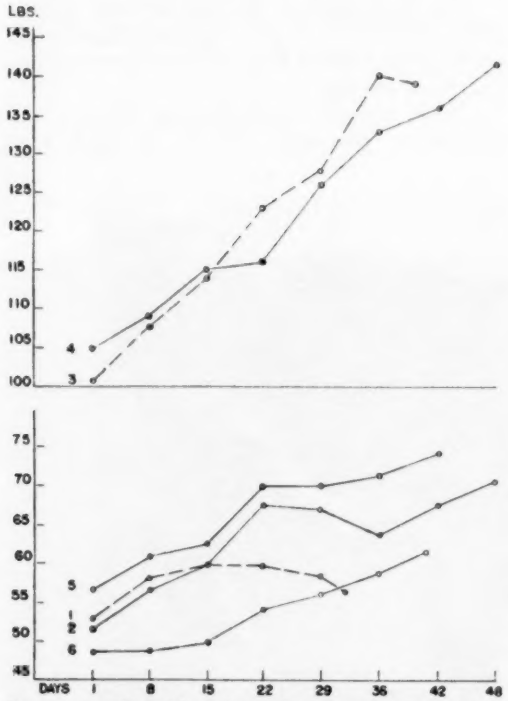


Fig. 7—Alpha-tocopherol-deficient calf D4 with lowered neck, extended head, and prominent suprascapula.

until the signs of deficiency had become pronounced (with exception of calf D4 which showed plasma *alpha*-tocopherol to be 125 μg./100 ml. on the 7th day). When the signs of deficiency were most severe, *alpha*-tocopherol unexpectedly appeared in the blood plasma in the following amounts: calf D1, 285 μg.; calf D2, 90 μg.; Calf D3, 120μg.; and calf D4, 30 μg. per 100 ml. of plasma (table 1).

Calf C5 had 90 μg. of *alpha*-tocopherol per 100 ml. of blood plasma the first day. This amount continuously increased until 1,310 μg./100 ml. of blood plasma was present on the forty-second day. Calf C6 had 350 μg. of *alpha*-tocopherol per 100 ml. of blood plasma the first day, none in its blood samples the seventh and fourteenth days, then a gradual increase to 690 μg. of *alpha*-tocopherol per 100 ml. of plasma on the forty-second day.

Calf D2, which was given four subcutaneous injections totalling 3,390 mg. of *alpha*-tocopherol acetate over a period of five days, did not show *alpha*-tocopherol in the blood plasma but calf D4 given three injections, totalling 2,500 mg. of *alpha*-tocopherol acetate over a period of five days, showed an increase of plasma *alpha*-tocopherol from zero to 135 μg./100 ml. (table 1).



Graph 1—Weight gains of alpha-tocopherol-deficient calves 1, 2, 3, 4, and control calves 5 and 6.

TABLE 1—Blood Plasma Alpha-Tocopherol Levels (μg./100 ml.)

Age (days)	Calf D1	Calf D2	Calf D3	Calf D4	Calf C5	Calf C6
1	0	0	0	0	90	315
7	0	0	0	125	225	0
14	0	0	0	0	348	0
21	0	0	0	0	348	540
28	285	0	0	0	825	405
33	75					
35		90	120	0	900	630
42		15	0	30	1310	690
44				0†		
45		*		30		
46		0		0**		
47		0**		120		
48		0		135†		
49		0†				
50		0†				
51		0				

* 890 mg. *alpha*-tocopherol acetate injected subcutaneously. ** 500 mg. *alpha*-tocopherol acetate injected subcutaneously. † 1,000 mg. *alpha*-tocopherol acetate injected subcutaneously.

Colostrum Alpha-Tocopherol.—The colostrum was defatted by removing cream in an ordinary cream separator. The defatted colostrum fed to the calves prior to starting the feeding of the synthetic milk contained the following amounts of *alpha*-tocopherol, in micrograms/100 ml.: calf D1, 527; calf D2, 90; calf D3, 280; calf D4, 295; calf C5, 180; and calf C6, 252.

Heart Rates Determined by Auscultation.—The average heart rates determined twice daily on all the calves for the first week were compared with the averages of the last week the calves were on experiment (graph 2). The percentage reduction in heart rates was as follows: calf D1, 30.7; calf D2, 16.4; calf D3, 5.4; calf D4, 25.6; calf C5, 15.0; and calf C6, 9.7.

Electrocardiograms.—The average duration of the intervals, the cycles, rates and Bazett's K* in the electrocardiograms (ECGs) made on all the calves are listed in table 2.

The average potentials in the ECGs recorded from the control calves were as follows:** P_1, 0.148; R_1, 0.543; T_1, 0.23; P_2, 0.256; R_2, 0.488; T_2, 0.521; P_3, 0.201; R_3, 0.566; and T_3, 0.501 millivolts. The average potentials from the deficient calves

* Formula for correction for the rate of the heartbeat in measuring the Q-T interval in the electrocardiogram;

** P, Q, R, S, T are designations of waves, and 1, 2, 3, the leads, on the electrocardiogram.

were: P_1, 0.212; R_1, 0.672; T_1, 0.367; P_2, 0.264; R_2, 0.956; T_2, 0.433; P_3, 0.184; R_3, 0.788; and T_3, 0.332 millivolts.

The ST_3 segment in the ECG made from calf D3 at 38 days of age was slightly elevated. An elevation was present in the ST_2 segment in the ECG made from calf C6 at 40 days of age.

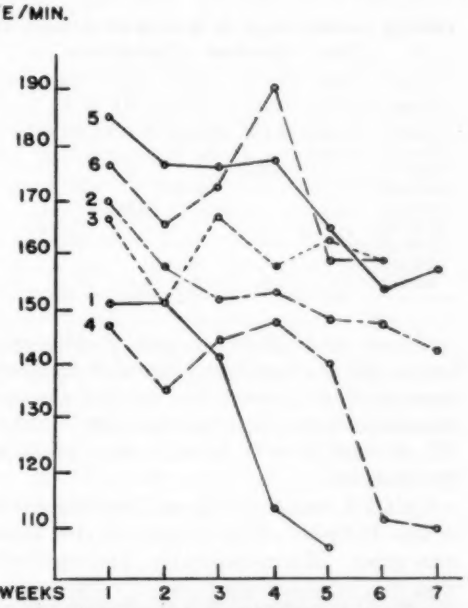
Urinalyses.—The color of the urine samples varied from a very pale yellow to cloudy yellow to dark yellow. The specific gravity varied from 1.003 to 1.030. For the most part, the urine reaction of all the calves was pH 6 with an occasional, noncorrelated variation to pH 7 to pH 8.

Albumin was strongly positive in the first urine samples collected from all of the calves but three days later all were negative. All of the subsequent urine samples were negative for albumin with the following exceptions: calf D1, twenty-fifth day, 2 plus; twenty-eighth day, 3 plus; thirty-first and thirty-third days, 4 plus; calf D2, twenty-fourth, twenty-seventh, thirty-ninth, and forty-second days, 1 plus; calf D4, fortieth day, 1 plus; and calf C6, eleventh and thirty-fifth days, 1 plus.

TABLE 2—Heart Rates, Bazett's K, P-R, Q-T, and Cycle Intervals of the Control and Deficient Calves

Calf No.	Age (days)	P-R* (sec.)	Q-T* (sec.)	Cycle (sec.)	Rate (min.)	Bazett's K**
D1	27	0.16	0.36	0.68	89.0	0.437
	31	0.14	0.28	0.60	100.0	0.359
	33	0.16	0.36	0.78	82.0	0.420
Ave.		0.153	0.333	0.67	90.3	0.406
D2	16	0.14	0.36	0.72	83.0	0.426
	23	0.08	0.28	0.52	115.0	0.387
	30	0.12	0.30	0.72	83.0	0.355
	38	0.16	0.36	0.72	83.0	0.426
	44	0.16	0.26	0.60	100.0	0.334
	48	0.16	0.36	0.64	93.0	0.450
Ave.		0.143	0.353	0.653	92.8	0.437
D3	16	0.12	0.28	0.64	93.0	0.350
	23	0.16	0.28	0.64	93.0	0.350
	30	0.12	0.28	0.48	125.0	0.401
	38	0.16	0.36	0.64	93.0	0.450
Ave.		0.14	0.30	0.60	101.0	0.387
D4	15	0.12	0.24	0.50	120.0	0.342
	22	0.14	0.32	0.80	75.0	0.356
	29	0.10	0.24	0.36	168.0	0.400
	31	0.14	0.32	0.72	83.0	0.378
	43	0.14	0.32	0.76	79.0	0.364
	47	0.10	0.32	0.66	91.0	0.394
Ave.		0.123	0.293	0.633	102.6	0.372
C5	10	0.10	0.24	0.54	111.0	0.326
	17	0.11	0.32	0.58	103.0	0.417
	24	0.12	0.28	0.60	100.0	0.359
	32	0.10	0.32	0.86	70.0	0.346
	38	0.12	0.30	0.72	83.0	0.355
Ave.		0.11	0.292	0.66	93.4	0.360
C6	8	0.10	0.32	0.58	103.0	0.417
	18	0.11	0.24	0.44	136.0	0.360
	22	0.11	0.28	0.46	129.0	0.412
	30	0.11	0.32	0.52	115.0	0.443
	36	0.12	0.28	0.56	107.0	0.375
	40	0.10	0.24	0.38	158.0	0.392
Ave.		0.108	0.28	0.49	124.0	0.401

* P, Q, R, S, T are designations of waves of the electrocardiogram. ** Formula for correction for the rate of the heartbeat in measuring the Q-T interval in the electrocardiogram.



Graph 2—Heart rates as determined by twice daily auscultation of alpha-tocopherol-deficient calves 1, 2, 3, 4, and control calves 5 and 6.

Occult blood was present in the first urine samples from each calf. This was associated with urine samples becoming contaminated with blood from the navel cords. By the fifteenth day, all samples were negative. All the urine samples then remained negative for occult blood with the exception of those taken from calf D1 on the twenty-eighth, thirty-first, and thirty-third days.

The urinary excretion of creatine, as measured by the ratio

$$\frac{\text{creatine}}{\text{creatine plus creatinine}},$$

was followed during the period of the experiment. Comparing the first twenty-one days of the experiment with the subsequent days, the percentage of decrease in the creatine excretion was found to be as follows: calf D1, 27.9; calf D2, 15.4; calf D3, 6.8; calf D4, 18.5; control calves C5, 27.4; and C6, 27.6. The average decrease among the deficient calves was 17.1, while

the average decrease of the controls was 27.5 (table 3). All urine samples were negative for acetone, diacetic acid, glucose, and bile.

TABLE 3—Averages of Ratios of Urinary Creatine: Creatine + Creatinine

Age (days)	Calf D1	Calf D2	Calf D3	Calf D4	Calf C5	Calf C6
1 to 21	0.627	0.537	0.174	0.427	0.434	0.474
22 to 31	0.452					
22 to 51		0.454				
22 to 40			0.162			
22 to 51				0.348		
22 to 48					0.315	
22 to 43						0.343
Reduction (%)	27.9	15.4	6.8	18.5	27.4	27.6

Gross and Microscopic Pathology.—Gross and microscopic muscular dystrophic changes were observed in all 4 of the calves maintained on the deficient diet (table 4). All skeletal muscle lesions were bilaterally symmetrical.

Calf D1 was the only calf having changes in the kidneys. The cortex of the kidneys was grey. Microscopically, the epithelium of the tubules was flattened. Many foci of masses of leukocytes were present in the cortex capillaries.

No muscular dystrophic changes were observed in the control calves.

DISCUSSION

Signs of muscular dystrophy appeared in 4 calves between the ages of 12 and 24 days, on a tocopherol-deficient, synthetic milk diet. The control calves did not demonstrate signs of muscular dystrophy at any time during the feeding period. Muscular weakness developed progressively in the 2 Jersey calves but was not apparent in the 2 Holstein-Friesian calves until other signs had become quite pronounced. Winged scapulae were prominent in the Holstein-Friesians but not in the Jersey calves. The extreme relaxation and lowering of the fetlocks in the Jersey calves were not apparent in the others. Difficulty in sucking the rubber nipple was observed in all of the deficient calves.

These signs were similar to those observed in the spontaneous skeletal muscle

TABLE 4—Gross and Microscopic Muscular Dystrophic Lesions in the Alpha-Tocopherol Deficient Calves

Calf No.	Muscle	Gross lesions	Microscopic lesions
D1	Vastus medialis	Extensive	Extensive
	Supraspinatus	Extensive in ventral portion	Extensive
	Triceps	Distinct near olecranon	Extensive
	Rhomboideus	Distinct along posterior border	Extensive
	Infraspinatus	Extensive in medial portion	Extensive
	Trapezius	None	None
	Longissimus dorsi	Pale internal portion	Early stages of degeneration
	Gastrocnemius	None	Early stages of degeneration
	Tongue	Pale	Normal
	Heart	None	Normal
D2	Supraspinatus	Pale areas	Extensive in portion of sections
	Ulnaris lateralis	Extensive	
	Superficial digital flexors	Pale areas	
	Deep digital flexors	Extensive	
	Longissimus dorsi	Pale in medial portion	None
	Heart	None	None
	Tongue	Pale	None
D3	Triceps brachii	Pale	None
	Rhomboideus	None	Early stages
	Rectus femoris	None	Fiber bundles separated increase in nuclei
	Gastrocnemius	None	None
	Infraspinatus	None	None
	Heart	Pale left ventricle	None
	Tongue	Pale	None
D4	Supraspinatus	Pale areas	Fiber swelling and fragmentation
	Triceps	Pale near olecranon	
	Extensor carpi radialis	Extensive in internal portion	Extensive
	Superficial gluteal	Extensive	
	Biceps femoris	Extensive	
	Semitendinosus	Extensive	Extensive
	Rectus femoris	Small areas	Extensive
	Tongue	None	Small areas showing fiber fragmentation
	Heart	None	None

form of "white muscle" disease observed in beef calves in Montana. However, while approximately 90 per cent of the latter have the cardiac type of this disease resulting in sudden death, none of the calves in this experiment developed cardiac lesions. Blaxter *et al.*[22] reported extensive cardiac involvement in a calf maintained on their vitamin E-deficient rations. Culik *et al.*,[10] using a deficient, synthetic milk similar to the one used in this experiment, reported both skeletal and cardiac symptoms and lesions in lambs.

The loss of weight during the last ten days of life in calf D1 and the last four days in calf D3 appeared to be due to the effects of the tocopherol-deficient diet, although the deficient diet had no effect on the weight gains of the other 2 calves.

A distinct lowering of the heart rate was noted in 2 of the deficient calves, D1 and D2, coincidental with the appearance of signs of muscular dystrophy.

The ECG tracings of the deficient calves had an average P-R interval of 0.139 seconds and an average Q-T interval of 0.319 seconds (table 2). The average P-R interval of the control calves was 0.109 seconds and the Q-T interval, 0.286 seconds. Bacigalupo, Alfredson, Luecke, and Thorp[32] noted an increased P-R interval in ECGs made on vitamin E-deficient lambs. Gullickson and Calverly[18] observed an increased P-R interval in the ECGs made on a cow fed a vitamin E-deficient ration.

The potentials of P_1, R_1, T_1, P_2, and R_3 waves of the ECGs of the deficient calves were greater than those of the control calves. The potentials of the T_2, P_3, and T_3 waves of the control calves were greater than those of the deficient calves.

All of the ECG readings of the deficient and control calves were within the normal limits described by Lepeschkin.[33] The control calves had a tendency to a little less change in the various complexes than the deficient calves.

On the thirtieth day, calves D2 and D3 demonstrated signs of muscular dystrophy to such a degree they were able to arise

Fig. 8—Section of infraspinatus muscle of alpha-tocopherol-deficient calf D1 showing muscle fiber degeneration and nuclear invasion. Hematoxylin-eosin. x 100.

only with difficulty or assistance. A gradual four- to six-day improvement which occurred on the thirtieth day in calves D2 and D3 could have been due to the compensatory action of the musculature not so severely involved. This improvement was also associated with the sudden appearance of detectable amounts of *alpha*-tocopherol in the blood plasma.

Plasma *alpha*-tocopherol determinations remained negative in the deficient calves until signs of muscular dystrophy became severe. The unexpected appearance of *alpha*-tocopherol in the blood plasma of calves D1, D2, and D3 (table 1) corresponded with their weight losses. It also corresponded with the temporary recoveries noted in calves D2 and D3. Calf D4 did not show weight loss or temporary recovery, but signs of muscular dystrophy were advanced at the time of the appearance of the plasma *alpha*-tocopherol. The mechanisms involved with the sudden appearance of *alpha*-tocopherol in the blood plasma of the deficient calves are not explainable from observations made in this experiment.

The administration of *alpha*-tocopherol acetate to calf D2, starting on the forty-fifth day, did not result in an increase of plasma *alpha*-tocopherol (table 1), although immediate and definite improvement was observed. The administration of *alpha*-tocopherol acetate to calf D4, starting the forty-fourth day, resulted in a rise from nondetectable amounts of plasma *alpha*-tocopherol to 135 μg. per 100 ml. on the forty-eighth day and an immediate definite improvement.

No plasma *alpha*-tocopherol was found in the blood taken from calf C6 on the seventh and fourteenth days (table 1). This drop of plasma *alpha*-tocopherol corresponded with the period of illness the first ten days of the calf's life. This would seem to indicate that *alpha*-tocopherol was not absorbed from the digestive tract during periods of prolonged diarrhea.

The defatted colostrum fed to calves D1, D2, D3, and D4 contained 90 to 527 μg. of *alpha*-tocopherol per 100 ml. *Alpha*-tocopherol was not found in the calves' blood. The first day, calves C5 and C6 were fed colostrum which contained 180 and 252 μg. of *alpha*-tocopherol per 100 ml. The first plasma *alpha*-tocopherol determinations on calves C5 and C6 revealed 90 and 315 μg. per 100 ml. (table 1). The only correlating factor was that calves D1, D2, D3, and D4 were received from one

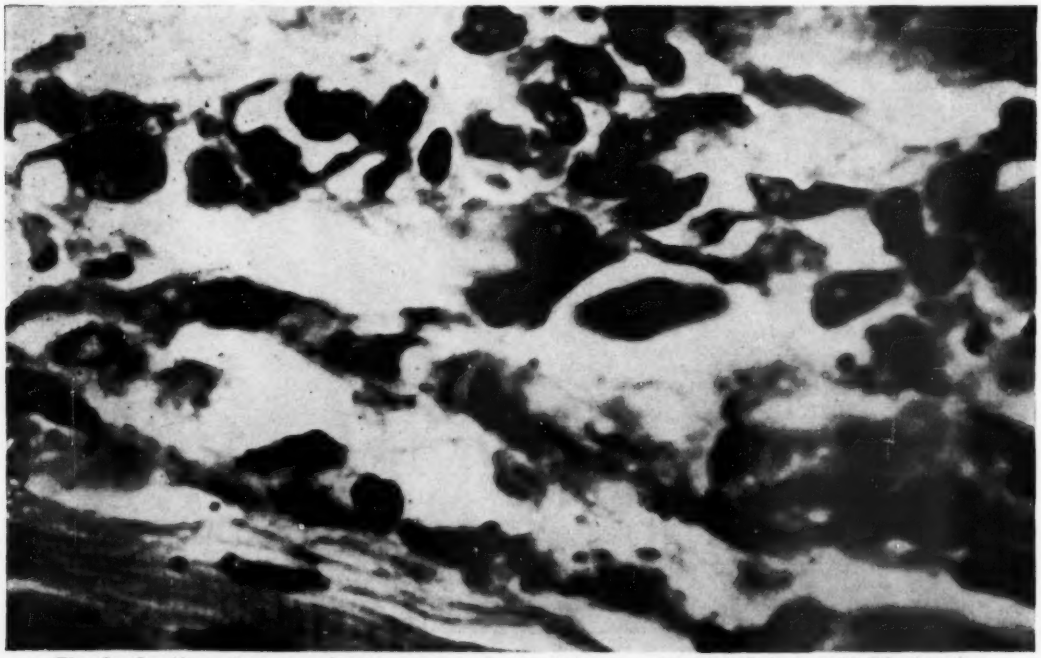


Fig. 9—Section of supraspinatus muscle from alpha-tocopherol-deficient calf D2 showing muscle fiber degeneration and nuclear invasion. Hematoxylin-eosin. x 440.

dairy and calves C5 and C6 from another.

An increasing amount of albumin appeared in the urine of calf D1 simultaneously with the increased intensity of the signs of muscular dystrophy, and it was the only calf to show gross pathological changes in the kidneys on autopsy.

Occult blood was detected in the urine of calf D1 coincidentally with the presence of muscular dystrophy, but this was not observed in the other calves.

The gross and microscopic pathology observed in the kidney is quite similar to the changes occasionally observed in field cases of white muscle disease in Montana beef calves.[2] Occasionally, the kidneys observed in field cases present a pale cortex similar to the cortex of the kidney observed in calf D1. Microscopically, the field cases show degeneration of the tubule epithelium, engorgement of the capillaries with leukocytes and erythrocytes, foci of leukocytes in the interstitial tissue, and distended thin-walled tubules. Occasionally, the cortex of the kidney in field cases is petechiated or engorged. Blaxter *et al.*[22] reported hemorrhagic infarcts on the cortex of the kidneys taken from 1 of their vitamin E-deficient calves. Welch and Jungherr[34] reported pathological lesions, similar to those described here as frequently occurring in lambs that died from white muscle disease. Metzger and Hagan[35] reported an enlarged and very white kidney taken from a lamb. Microscopic section of this kidney revealed an acute fatty degeneration of the epithelium of the convoluted tubules and distention of the glomeruli. Metzger and Hagan refer to Hobmaier's report[36] in which considerable attention was paid to the pathological changes of the kidneys of lambs with a disease similar to the so-called "stiff lamb" disease.

For comparative purposes, the ratios of urinary creatine to creatine plus creatinine the first twenty-one days were compared with the last days of life. This period was selected because on the twenty-first day signs of muscular dystrophy were apparent in the 4 deficient calves in varying degrees. Calf D1, which was severely affected with muscular dystrophy, showed approximately the same reduction in the ratio as the control calves but calves D2, D3, and D4 had a less reduction (table 3). These findings would indicate, as determined by Blaxter *et al.*,[22] that creatinuria is not necessarily diagnostic of advanced nutritional muscular dystrophy in calves.

The gross lesions observed in the skeletal muscles of the deficient calves were similar to those described by Vawter and Records.[1]

The muscular dystrophic changes (fig. 8) observed in the deficient calves were fragmentation and hyalinization of the muscle fibers. Nuclear invasion of the sarcolemmal sheaths was often observed before much change had occurred in the adjacent muscle fibers. In some muscles, the degeneration was so extensive as to cause complete disappearance of muscle fibers and occasionally of entire muscle bundles. Extensive nuclear invasion replaced the degenerated muscle fibers. The bulk of the dense mass of nuclei was identified as sacrolemmal nuclei (fig. 9). A few scattered groups of histiocytes were observed, and an occasional lymphocyte was present, while granulocytes and giant cells were notably absent. Muscle striations remained visible until the sarcoplasm disappeared. Occasional small foci resembling calcification were observed in some of the slides. Hadlow,[37] upon examination of sections prepared from the deficient calves, observed an apparent endothelial swelling and proliferation causing some narrowing of the lumens of many capillaries and arterioles.

The tissue changes observed in the slides from the calves on this experiment were similar to those observed in the muscle tissues taken from field cases of white muscle disease of both calves and lambs[2] and to those described by MacDonald *et al.*[24] Polymorphonuclear leukocytes and giant cells observed by Vawter and Records[1] were not observed in the sections prepared from the calves of this experiment nor in the sections taken from spontaneous field cases of white muscle disease in calves and lambs in Montana.

SUMMARY

Signs of deficiency and skeletal muscle lesions similar to those observed in field cases of the skeletal form of "white muscle" disease were produced in 4 calves maintained on a synthetic tocopherol-deficient milk. Two calves maintained on the same milk that was supplemented with 148 mg. of *d-alpha*-tocopherol acetate daily did not develop these signs or lesions.

Weight gains of the calves were not affected until signs of deficiency were advanced.

Body temperatures, number of erythrocytes and leukocytes, and amounts of hemoglobin were not altered in the tocopherol-deficient calves.

Traces of *alpha*-tocopherol appeared in the blood plasma of all 4 of the deficient calves coincidentally with the occurrence of the most severe signs of deficiency.

Electrocardiograms of the deficient calves had a longer P-R and Q-T interval than the control calves.

Albuminuria, and the presence of occult blood in the urine, together with macroscopic kidney changes were observed in 1 of the 4 deficient calves. Creatinuria was not a sign of *alpha*-tocopherol deficiency in the calves.

CONCLUSIONS

Tocopherol deficiency in calves produces signs and lesions similar to those of spontaneous "white muscle" disease in calves.

References

[1] Vawter, L. R., and Records, Edward: Muscular Dystrophy in Young Calves. J.A.V.M.A. *110*, (1947): 152-157.

[2] Unpublished data, Montana Veterinary Research Laboratory files.

[3] Goettsch, Marianne, and Pappenheimer, Alwin M.: Nutritional Muscular Dystrophy in the Guinea Pig and Rabbit. J. Exptl. Med., *54*, (1931): 145-165.

[4] Pappenheimer, A. M.: The Pathology of Nutritional Muscular Dystrophy in Young Rats. Am. J. Path., *15*, (1939): 179-184.

[5] Evans, H. M., Emerson, G. A., Telford, I. R.: Degeneration of Cross Striated Musculature in Vitamin E-Low Rats. Proc. Soc. Exptl. Biol. and Med., *38*, (1938): 625-627.

[6] Follis, Richard H., Jr.: The Pathology of Nutritional Disease. Charles C. Thomas, Springfield, Ill. (1948): 119-127.

[7] Pappenheimer, A. M.: Muscular Dystrophy in Mice on Vitamin E-Deficient Diet. Am. J. Path., *18*, (1942): 169-174.

[8] Willman, J. P., Asdell, S. A., Grams, W. T., Hagan, W. A.: The Stiff Lamb Disease. Proc. Am. Soc. Anim. Prod. (1931): 231-234.

[9] Marsh, Hadleigh: Diseases of Young Lambs. J.A.V.M.A., *81*, (1932): 187-194.

[10] Culik, R., Bacigalupo, F. A., Thorp, F., Jr., Luecke, R. W., Nelson, R. H.: Vitamin E Deficiency in the Lamb. J. Anim. Sci., *10*, (1951): 1006-1016.

[11] Shimotori, N., Emerson, G. A., and Evans, H. M.: The Prevention of Nutritional Muscular Dystrophy in Guinea Pigs with Vitamin E: J. Nutr., *19*, (1940): 547-554.

[12] Goettsch, Marianne, and Pappenheimer, Alwin M.: *Alpha*-tocopherol Requirements in the Rat for Reproduction in the Female and Prevention of Muscular Dystrophy. J. Nutr., *22*, (1941): 467-476.

[13] Adamstone, F. B., Frider, J. L., and James, M. F.: Response of Swine to Vitamin E-Deficient Rations. Ann. N. Y. Acad. Sci., *52*, (1949): 234-242.

[14] Whiting, F., Willman, J. P., and Loosli, J. K.: Tocopherol Deficiency Among Sheep on Natural Feeds. J. Anim. Sci., *8*, (1949): 234-242.

[15] Willman, J. P., Loosli, J. K., Asdell, S. A. Morrison, F. B., and Olafson, Peter: The Prevention and Cure of Muscular Stiffness in Lambs. J. Anim. Sci., *4*, (1945): 128-130.

[16] Marsh, H.: Treatment of Stiff Limbs with Wheat Germ Oil. J.A.V.M.A., *108*, (1946): 256.

[17] Gullickson, T. W., Palmer, L. S., Boyd, W. L., Nelson, J. W., Olson, F. C., Calverly, C. E., and Boyer, P. D.: Vitamin E in the Nutrition of Cattle. I. Effect of Feeding Vitamin E-Poor Rations on Reproduction, Health, Milk Production, and Growth. J. Dai. Sci., *32*, (1949): 495-508.

[18] Gullickson, T. W., and Calverly, C. E.: Cardiac Failure in Cattle on Vitamin E-Free Rations as Revealed by Electrocardiograms. Science, *104*, (1946): 312-313.

[19] Latschar, C. E., Wise, G. H., Parrish, D. B., and Hughes, J. S.: Concentrations of Various Constituents in the Blood of Dairy Cows During Stages of Terminal Gestation and Initial Lactation. I. Effect of Prepartal Diet on Serum Tocopherols. J. Nutr. *38*, (1949): 503-516.

[20] Parrish, D. B., Wise, G. H., and Hughes, J. S.: The Properties of the Colostrum of the Dairy Cow. I. Tocopherol Levels in the Colostrum and in the Early Milk. J. Dai. Sci., *30*, (1947): 849-860.

[21] Parrish, D. B., Wise, G. H., Latschar, C. E., and Hughes, J. S.: Effect of Prepartal Diet of the Cow on Placental and Mammary Transfer of Tocopherols to the Calf. J. Nutr., *40*, (1950): 193-202.

[22] Blaxter, K. L., Watts, P. S., and Wood, W. A.: The Nutrition of the Young Aryshire Calf. 8. Muscular Dystrophy in the Growing Calf. Brit. J. Nutr., *6*, (1952): 125-144.

[23] Blaxter, K. L., and Wood, W. A.: The Nutrition of the Young Aryshire Calf. 9. Composition of the Tissues of Normal and Dystrophic Calves. Brit. J. Nutr., *6*, (1952): 144-163.

[24] MacDonald, A. M., Blaxter, K. L., Watts, P. S., and Wood, W. A.: Nutrition of the Young Aryshire Calf. 10. Histopathology of Muscular Dystrophy and Its Relation to Muscle Chemistry. Brit. J. Nutr., *6*, (1952): 164-169.

[25] Wiese, A. C., Johnson, B. Connor, Mitchell, H. H., and Nevans, W. B.: Synthetic Rations for the Dairy Calf. J. Dai. Sci., *30*, (1947): 87-94.

[26] Luecke, R. W., Culik, R., Thorp, F., Jr., Blakslee, L. H., and Nelson, R. H.: Riboflavin Deficiency in the Lamb. J. Anim. Sci., *9*, (1950): 420-425.

[27] Johnson, B. Connor, James, Marian F., and Krider, J. L.: Raising Newborn Pigs to Weaning Age on a Synthetic Diet with Attempt to Produce a Pteroyglutamic Acid Deficiency. J. Anim. Sci., *7*, (1948): 486-493.

[28] Quaife, Mary L., and Harris, Philip L.: The Chemical Estimation of Tocopherols in Blood Plasma. J. Biol. Chem., *156*, (1944): 499-505.

[29] Beeson, W. M., Pence, J. W., and Holm, G. C.: Urinary Calculi in Sheep. Am. J. Vet. Res., *4*, (1943): 120-126.

[30] Folin, O.: On the Determination of Creatinine and Creatine in Urine. J. Biol. Chem., *17*, (1914): 469-473.

[31] Quaife, Mary L.: Tocopherols (Vitamin E) in Milk: Their Chemical Determination and Occurrence in Human Milk. J. Biol. Chem., *169*, (1947): 513-514.

[32] Bacigalupo, F. A., Alfredson, B. V., Luecke, R. W., and Thorp, F., Jr.: Electrocardiographic Changes in Vitamin E-Deficient Lambs. Am. J. Vet. Res., *14*, (1953): 214-218.

[33] Lepeschkin, Eugene: Modern Electrocardiography. Vol. I. The Williams and Wilkins Co., Baltimore. (1951): 134-136.

[34] Welch, Howard, and Jungherr, Erwin: Unpublished data, Montana Veterinary Research Files. 1928-1930.

[35] Metzger, H. J., and Hagan, W. A.: The So-Called Stiff Lambs. Cornell Vet., *17*, (1927): 35-42.

[36] Hobmaier, M.: Die sogenannte Hemoglobinuria enzootica des Pferdes und ihr verwandte Krankheiten unserer Haustiere. Arch. f. wissensch. prakt. Tierheilk., *54*, (1926): 213.

[37] Hadlow, W. J., Rocky Mountain Laboratory, Hamilton, Mont.: Personal communication, 1953.

The Teeth of the Ox in Clinical Diagnosis
II. Gross Anatomy and Physiology

NORMAN L. GARLICK, D.V.M.

Tacoma, Washington

Part I of this series of papers which appeared in the April, 1954, issue of the AMERICAN JOURNAL OF VETERINARY RESEARCH *covered briefly the essentials of the histology and embryology of the teeth of cattle. The research background for the material herein presented was also given. A bibliography for suggested further study was included and will not be repeated here. Only actual references will be indicated. The material in this paper covers the essentials of gross anatomy and physiological response of teeth to environmental influences, knowledge of which is essential in rational diagnoses of dental anomalies and pathology.*

GROSS ANATOMY AND PHYSIOLOGY

Deciduous Dentition of the Ox.—The formula for the deciduous dentition of the ox is commonly expressed by veterinarians as follows (*see* note, introduction to part I):

$$2\left\{\text{Di}\,\frac{0}{4}\;\text{Dc}\,\frac{0}{0}\;\text{Dp}\,\frac{3}{3}\right\} = \text{Total } 20$$

Simply, eruption of the deciduous teeth takes place prior to birth or within the first three weeks of extra-uterine life. Therefore, the development of the deciduous teeth can be said to take place almost entirely during intra-uterine life. Some influences which tend to interfere with optimal dental development following birth have not been observed to affect deciduous teeth of cattle. Prior to birth, the placental membranes apparently are efficient in procuring necessary structural elements and screening out those substances which negatively influence the perfection of the permanent dentition.

The Deciduous Incisor.—Grossly, at birth, the enamel of the crown of the deciduous incisor tooth appears white and is translucent. The labial surface is slightly rough, and the tip of the tooth is quite sharp. The general appearance of the tooth is that of a shovel, with the blade narrowing distinctly at the gum line. The tooth is held loosely in the dental arch by the alveolar connective tissue which is closely attached to cementum, alveolar bone, and gingiva. Eruption of all deciduous teeth is relatively fast until they have taken their places in the dental arch. From that time on until they are lost, eruption is continuous, though at a very slow rate.

The Deciduous Premolar.—Most deciduous premolars are multicuspid and some are more complex, though all are smaller, than the permanent premolars which follow them. They have two divisions to the main pulpal portion, and some have several canals. For example, the third mandibular premolar has five pulp canals (fig. 1), each of which extends into the tip of the crown and terminates beneath a cusp. Between the medial and lateral cusps of this tooth, two extensive infundibula are present which extend all the way through the crown to the level of the root. Before eruption, the infundibula are partially filled with cement. This tooth does not resemble its successor, as might be supposed but, rather, the third mandibular molar tooth.

Physiological Response of Deciduous Teeth to Environment and Attrition.—As the deciduous teeth come into use, and eruption slowly continues, the appearance changes. The enamel is worn away from the incisal margins and tips of the cusps, exposing the dentine beneath. The rough enamel surfaces tend to wear smooth. As the teeth wear back, secondary dentine is deposited in the original pulpal portion, and the soft tissues gradually recede. The gingival margin gradually recedes, also, passing the cemento-enamel junction and exposing the roots of the incisor teeth at the neck. The gingival tissues remain firmly attached, however, and are usually the last to be loosened when the deciduous teeth are lost. Attrition may be at a rate which will wear away the functional crown

The author acknowledges the financial aid of the Aluminum Company of America whose research grant covered the major portion of the expense incurred in this investigation. However, the statements and conclusions expressed in this article are those of the author alone.

The author is engaged in veterinary consultation and research work in Tacoma.

entirely before a tooth is replaced by its successor. Deposition of secondary dentine is normally fast enough to prevent exposure of the pulpal portion of the tooth. At first the deciduous incisor teeth are close together but, as the alveolar process grows to make room for the permanent dentition, they are gradually spread apart and the tips of the teeth no longer afford protection to the necks. Forage, as it slides over the exposed dentine of the root, gradually cuts in from the side. A great percentage of deciduous incisors which no longer present a crown have actually been cut off below the cemento-enamel junction, the crown falling off as a whole (fig. 2). Attrition of mandibular premolar teeth may reach a point where anterior and posterior portions of the teeth may be separated entirely. This process is aided by root resorption.

Dental calculus is heavily deposited on the premolar teeth and appears brown to black. Forage pigments may adhere to the surfaces of the teeth and usually may be seen near the necks of the incisor teeth. The pigments can be removed from the surface with a scraper or emory cloth.

Permanent Dentition of the Ox.—The formula for the permanent dentition of the ox is commonly expressed by veterinarians as follows:

$$2\left\{\mathrm{I}\,\frac{0}{4}\,\mathrm{C}\,\frac{0}{0}\,\mathrm{P}\,\frac{3}{3}\,\mathrm{M}\,\frac{3}{3}\right\} = \text{Total } 32$$

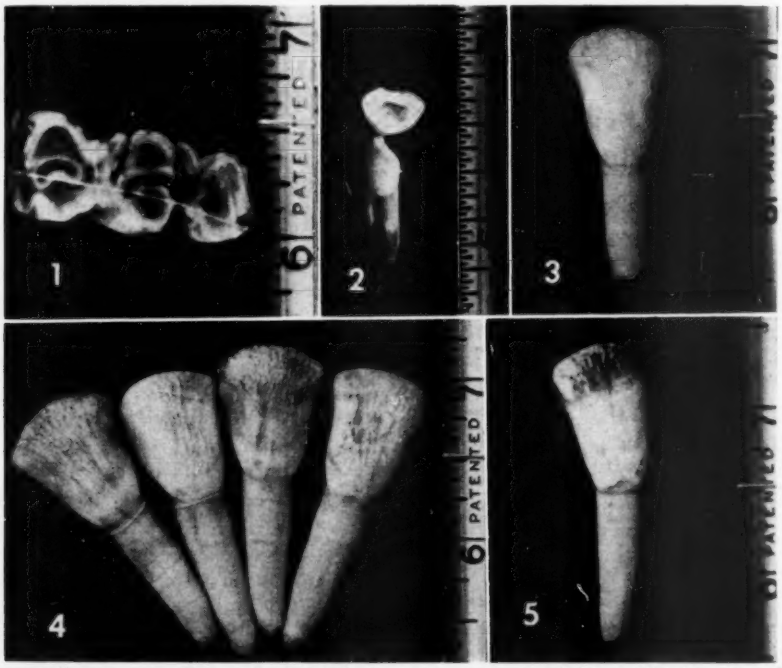


Fig. 1—The masticatory surface of the third deciduous mandibular premolar at time of shedding. The five primitive pulp canals are outlined by dark-staining secondary dentine. The two infundibula can be seen between the areas of secondary dentine, outlined by enamel, and containing black dental calculus.

Fig. 2—A deciduous incisor tooth removed just prior to shedding. Forage-caused abrasion at the cervix has nearly severed the tooth at that point. Root resorption is well advanced.

Fig. 3—A newly formed second incisor tooth removed immediately prior to eruption. The crown is complete, while the tip of the root is still actively forming.

Fig. 4—Four normal incisor teeth picked at random and removed at the time they were coming into wear or shortly thereafter. They show the corrugated vertically grooved appearance of newly formed incisor teeth, accentuated by the presence of surface pigments.

Fig. 5—An incisor tooth removed at that stage of eruption when about one third of the crown is showing above the gingival margin. The stains at the tip of the tooth have invaded the soft remnants of the epithelial attachment and are due to disappear as it peels off.

The chronology of eruption of the permanent dentition as given by Sisson [1] is quoted as follows:

Permanent:

First incisor (I 1)	1½ to 2 years
Second incisor (I 2)	2 to 2½ years
Third incisor (I 3)	3 years
Fourth incisor (I 4)	3½ to 4 years
First cheek tooth (P 1)	2 to 2½ years
Second cheek tooth (P 2)	1½ to 2½ years
Third cheek tooth (P 3)	2½ to 3 years
Fourth cheek tooth (M 1)	5 to 6 months
Fifth cheek tooth (M 2)	1 to 1½ years
Sixth cheek tooth (M 3)	2 to 2½ years

The eruption of the permanent teeth is subject to great variation. The above figures are the average of observations of improved breeds under favorable conditions.

The progression of eruption of the permanent cheek teeth in the maxilla is (using their numerical identification) 4, 5, 6, 1, 2, 3. The progression of eruption of the permanent mandibular cheek teeth is 4, 5, 6, 2, 1, 3. The mandibular cheek tooth slightly precedes its numerical counterpart in the maxilla, with the exception of the first premolar which may follow the upper. Variations in time of eruption are also considerable, occasionally, between the two individuals in any given pair. As Sisson points out, great variations occur in the chronology of permanent dental eruption. In cases of variation, it has been found that the tooth erupts at a later time than that shown in his schedule. Therefore, his table is considered the optimum and not a universal average.

The Permanent Incisor Teeth.—The usual number of permanent incisor teeth is eight but has been observed by the author to vary from six to nine. The newly erupted permanent incisor is from 1¼ to 2 in. in length from the apex of the root to the incisal margin. The central pair is usually the largest, the size decreasing with each pair as they form laterally. Occasionally, the second pair is the largest.

At the time of eruption, the enamel of the crown is practically complete (fig. 3). The labial surface is rough and vertically grooved, reflecting the corrugated contour of the dentino-enamel junction (fig. 4). Huidekoper,[2] in describing the permanent incisor teeth of the ox, states as follows:

> The external or under face is convex in both directions; it is of a milky-white color and is striped with little longitudinal ridges and gutters, which become polished smooth with age.

The grooves are more prominent in beef than in dairy breeds. The lingual surface is relatively smooth, but undulates from side to side. The incisal margin is relatively sharp and somewhat rough. The enamel is considerably thicker on the labial side than on the lingual. The crown presents a dull appearance due to the persistence of the soft remnants of the enamel organ and may even be a light yellow color (fig. 5).

Physiological Response of Permanent Incisor Teeth to Environment and Attrition.—As the epithelial attachment peels off the incisor, the hornified outer layer of the enamel cuticle usually absorbs forage and other pigments on the exposed surfaces which present a very light to dark brown color (fig. 6 and 7). The stains are most pronounced in the depths of the vertical grooves on the labial surface, and serve to accentuate the corrugated pattern of the enamel surface. The pigments sometimes penetrate through the cuticle and become impregnated into the terminal portions of the enamel prism area which is more poorly calcified than the deeper enamel (fig. 8). The stains can be removed with a scraper or emory cloth (fig. 9). The pigments persist for a variable period of time until, in the normal process of attrition, the stained structures are worn away. The cervical portion may retain the stain for many years.

As attrition continues, the vertical grooves become more shallow and the enamel becomes a lustrous translucent white. In general, the longer the cuticle of the enamel persists, and the longer the tooth retains its original vertically corrugated appearance, the higher the quality of the structure. The grooves on the fourth incisors may never be completely obliterated.

The incisal margin of the tooth soon wears to expose the primary dentine. The dentine wears slightly faster than the enamel, leaving the free edges of the latter slightly elevated. The tooth continues to wear and the secondary dentine is exposed. (As mentioned before, the secondary dentine is that formed within the primary pulpal portion of the tooth during the functional period to prevent exposure of the sensitive tissues.)

The occlusal surface of the incisor may remain relatively level or it may gradually

assume a cupped appearance. In the latter case, the enamel is considerably harder than the dentine. This phenomenon indicates a tooth of better than average quality or softer than average dentine. It is not a pathological process.

The term "occlusal surface," when used in conjunction with the description of an incisor tooth, requires an explanation since the incisors of the ox seldom serve the function of incision. They are set loosely in the alveolus and, when the mouth is closed, come in intimate contact with the tough dental pad of the upper jaw. Thus, there is an occlusion of the useful surfaces of the teeth, the food being held by this union and torn from its source. Therefore, the function of the incisor teeth of the ox is one of prehension dependent upon occlusion, and the useful surface can be termed the occlusal surface. The labial edge of the occlusal surface occasionally serves the purpose of incision and, therefore, can be referred to as the incisal margin.

The occlusal surface of the incisor, at about 7 years of age, is made up largely of dentine with a thin margin of enamel at the lingual edge and a thicker layer on the labial. The medial and lateral margins are protected by a layer of enamel which runs the breadth of the tooth in approximately a straight line. In the process of prehension, forage has a tendency to be drawn over the occlusal surface in a constant forward direction; thus the central dentinal portion will wear away at a faster rate than will the medial and lateral enamel margins (fig. 10) which will cause the cupped appearance. This is the opposite pattern of wear to that presented in certain pathological conditions that will be described later.

As the incisor tooth continues to wear, eruption continues, maintaining the occlusal pressure between incisor and dental pad. This rate of eruption is fairly constant, though slightly faster in beef breeds than in dairy animals. It is constant enough that it can be used to closely approximate the age of older animals as will be shown later. The gingival attachment gradually recedes as eruption continues until it is well down on the root portion of the tooth (fig. 11).

As the wider portions of the tips of the incisor teeth wear away, a space develops between them exposing the gingiva to traumatic influences. Abrasion begins on the necks of the teeth (fig. 11). When the dentinal portion of the roots extends above the gum line, the rate of wear at that point increases, and abrasional constrictions occur at the cervix stimulating secondary dentine formation. If the crown is severed from the root, as sometimes happens, it is found that the pulp has receded and is adequately protected by secondary dentine.

Concurrent with the trauma of the gingiva mentioned above, and with advancing age, increased recession of the periodontal

Legends for Illustrations on Opposite Page

Fig. 6—The second incisors from a 2½-year-old Hereford steer which have been in wear for a short time. The last remnants of the epithelial attachment are still present near the gingival margin.

Fig. 7—The first and second incisor teeth from a normal Hereford steer almost 3 years of age. Foreign pigments have invaded the outer layer of the enamel cuticle.

Fig. 8—The second incisor teeth of a normal 3-year-old Hereford steer. Pigments have deeply stained the enamel cuticle and have invaded the terminal portions of the enamel. At the edge of the tooth on the right, a small area has been cleaned with emory cloth to reveal the extremely hard enamel underlying the stained layers.

Fig. 9—The same teeth as shown in figure 11 after one of them has been cleaned with an emory cloth.

Fig. 10—An incisor tooth from a normal aged cow showing the cupped wear which is often present.

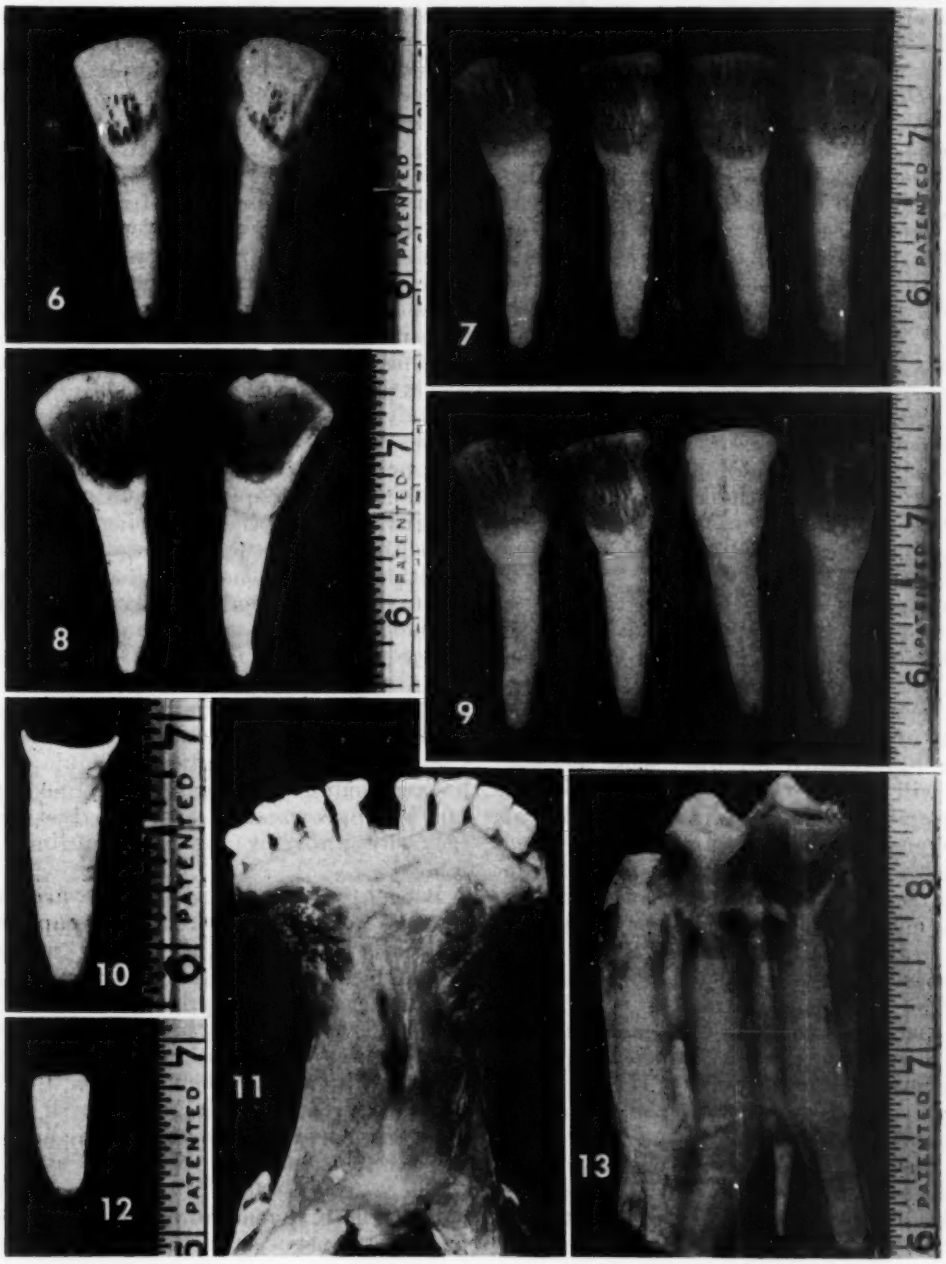
Fig. 11—The normal incisor teeth of a Hereford cow approximately 9 years of age. The gingival margin has receded well beyond the junction of root and crown. Abrasive influences have changed the appearance as evidenced by the deep notch in the side of the root of the right central incisor.

Fig. 12—The persisting portion of an incisor tooth from a normal older animal after the crown had worn away as a result of normal attrition and abrasion.

Fig. 13—The lateral surface of the third mandibular molar (sixth mandibular cheek tooth). Note the accessory root which occurs occasionally.

membranes takes place. Following this there is a recession of the border of the alveolar bone, lessening the depth of the alveolus and hastening loss of the tooth. That this process is partially dependent upon age is apparent in that teeth worn to the gum line prematurely as a result of fracture or pathology are not lost. Their roots persist until somewhere near the time when they would have been lost in the ordinary physiological progression of events.

As the animal increases in age, the tooth structure slowly loses moisture and a contraction takes place. As a result, the inelastic enamel begins to demonstrate fissures on the labial surface which take a vertical pattern. These may appear at any time from the age of 3 years on. The fissures



follow the direction of structural weakness in the enamel, fracturing the interprismatic substance. As was already explained, in the formation of enamel the prisms arise at an angle to the dentino-enamel junction and are largely directed toward the incisal margin. Thus, a horizontal fracture would of necessity break many enamel prisms while a vertical fracture would have to break few, if any. The presence of physiological enamel fissures is important in varying the pattern of pathological dental lesions as will be pointed out later.

The Ultimate Fate of the Permanent Incisor Teeth.—Since there is considerable difference in the fate of the incisor teeth in beef breeds as compared with dairy animals, they will be considered separately.

1) Beef Breeds.—Usually at the age of 7 years, the gingival margin recedes to a point where the roots of the central pair of incisors are exposed, recession of the periodontal connective tissue accelerates, the margins of the bony portion of the alveolus resorb, and the incisors become more loose. At the age of 8 years, the roots of the second pair of incisors are visible. The roots of the third and fourth pairs are in view by the end of the ninth year. This progression is subject to considerable variation, but the above is considered the average.

The natural tendency of the incisor to pry itself out of the alveolus during use is accelerated as the supportive tissues regress and as eruption continues. The leverage exerted is in direct proportion to the length of the tooth. Therefore, the tooth which has resisted attrition most successfully will require the most support. When the supporting tissues can no longer resist the leverage effect, they are torn away and the teeth are lost, usually in the order in which they erupt. The teeth that have worn faster maintain adequate support and are worn to the gum line, the entire functional crown being lost to normal abrasion and attrition. If this happens, the root of the tooth persists in the alveolus as a smooth slightly rounded knob of dentine (fig. 12). The primitive pulpal portion is largely, if not entirely, obliterated by the deposition of secondary dentine.

As was previously pointed out, the crown may be severed from the root at the cervix by the abrasive action of forage at that point, in which case the root will persist in the alveolus. Once a tooth has worn to the gum line it will persist for many years, as practically all functional stresses have ceased. It will continue to wear as the processes of dental eruption and alveolar regression continue. Roots as short as one third of an inch have been removed from aged subjects.

2) Dairy Breeds.—The processes of eruption and supportive tissue regression are slower in the dairy breeds. At the age of 8 years the roots of the central pair of incisors usually become visible, with the roots of the second pair showing up during the ninth year. By the end of the tenth year, the roots of the third and fourth pairs are showing. This gives the incisor teeth at least a year more of adequate support than is found in the average beef animal. As a result, leverage stress is reduced, there is more wear on the crown, and the number of teeth lost is considerably less than in the beef breeds. Also, the tendency for forage stresses to sever the tooth at the cervix is less evident.

The presence, in either dairy or beef groups, of persistent crowns with considerable portions of the roots exposed is an indication of high quality teeth, an exceptionally fast eruption rate, increased rate of periodontal tissue regression, decreased attrition and abrasion due to forage and pasture soil variations, or any combination of the four.

Age Approximation by the Appearance of the Permanent Incisor Teeth.—Up to the fourth year, the age of an animal can be closely approximated by applying a knowledge of the eruption schedule. Following completed primary eruption, ages can be approximated, but with less accuracy, by applying the data outlined in table 1.

Because of the considerable variations that occur, this chronology of age approximation is offered only as a rough estimate, although it is considered an improvement over other existing approximation tables.

The Permanent Cheek Teeth.—Since the examination of the cheek teeth in the living animal is limited by their location, this description gives only the groundwork necessary to recognize patterns of wear and the usual appearance.

There are usually 24 permanent cheek teeth. The first mandibular premolar may be absent on one or both sides in a small

percentage of animals, even though the analogous deciduous tooth may have been present.

The Mandibular Cheek Teeth.—The lower cheek teeth are bi-rooted, though they may commonly demonstrate one or more rudimentary accessory roots (fig. 13). The teeth increase in size, markedly, from the first to the sixth in all dimensions except mediolaterally (fig. 14 and 15). When in full wear, approximately four fifths of the anatomical crown is visible above the gingival margin on the first four; one half on the fifth; and only one third on the sixth (fig. 16). Root portions, particularly on the last three, are still actively forming when the teeth are in full wear. Infundibula are shallow in the premolars, but two, well-developed, are present in each molar. Small accessory cusps and enamel irregularities, which may be present, particularly on the lateral margins of the molar teeth, present wide variations in development. The convolutions of the enamel surfaces are complex, though constant enough to bear description. However, they are relatively unimportant and will not be described in detail. The enamel surfaces present the same corrugated pattern of vertical grooves that are so clearly seen on the permanent incisors but are harder to demonstrate because of the early acquired heavy deposits of dental calculus and pigments on all but the masticatory surface and the margins of the cusps.

The Maxillary Cheek Teeth.—The permanent maxillary cheek teeth vary from the mandibular in many ways. The upper cheek teeth are tri-rooted; accessory roots are rare; and variations in size from the first to the sixth are evident but not to the extent reached in the lower teeth (fig. 14). The main variation is in the length, with a slightly greater portion of the anatomical crown lying below the gingival margin in the early functional period (fig. 16). Again, it is the sixth tooth which has the greatest percentage of the anatomical crown within the alveolus. Each premolar presents one large well-developed infundibulum, while the molars each present two. Accessory cusps may be present, particularly on the medial margins of the molars where they are practically constant.

Opposition of the Permanent Cheek Teeth.—The cheek teeth do not lie in perfect opposition to their numerical counterparts in the opposite jaw (fig. 14 and 16). This provides an explanation of physiological and pathological patterns of attrition. The movements of the mandible of the ox are restricted largely to lateral, depression, and elevation. Protrusion and retraction are so slight that functionally they can be considered nonexistent. Therefore, the opposition of the cheek teeth remains constant, subject only to the variations occasioned by attrition. Because the dental arch of the mandible is much narrower than that of the maxilla, true occlusion of the cheek

TABLE 1—Data for Determining the Approximate Age of Beef and Dairy Cattle by the Appearance of the Permanent Incisor Teeth

BEEF BREEDS
4 years—Very little wear is apparent on the tips of the fourth pair of incisors.
5 years—Primary and secondary dentine are seen on the occlusal surface of the fourth pair. About one fourth of the original functional crown has worn away.
6 years—About one third of the original functional crown has worn from the fourth pair. The occlusal surface has reached its maximum anteroposterior diameter on the other three pairs.
7 years—The roots of the first pair become visible above the gingival margin.
8 years—The roots of the second pair become visible above the gingival margin. Loss of teeth may occur.
9 years—The roots of the third and fourth pairs become visible above the gingival margin. More teeth may be missing, and the persisting crowns are very short.
10 years and beyond—No close approximation can be made.

DAIRY BREEDS
4 years—Very little wear is apparent on the tip of the fourth pair of incisors.
5 years—Primary dentine can be seen on the occlusal surface of the fourth pair.
6 years—Primary and secondary dentine can be seen on the occlusal surface of the fourth pair. About one fourth of the original functional crown has been worn away.
7 years—About one third of the original functional crown of the fourth pair has been worn away.
8 years—The roots of the first pair of incisors become visible above the gingival margin.
9 years—The roots of the second pair of incisors become visible above the gingival margin.
10 years—The roots of the third and fourth pairs become visible above the gingival margin. The persisting crowns are quite short.
11 years and beyond—No close approximation can be made.

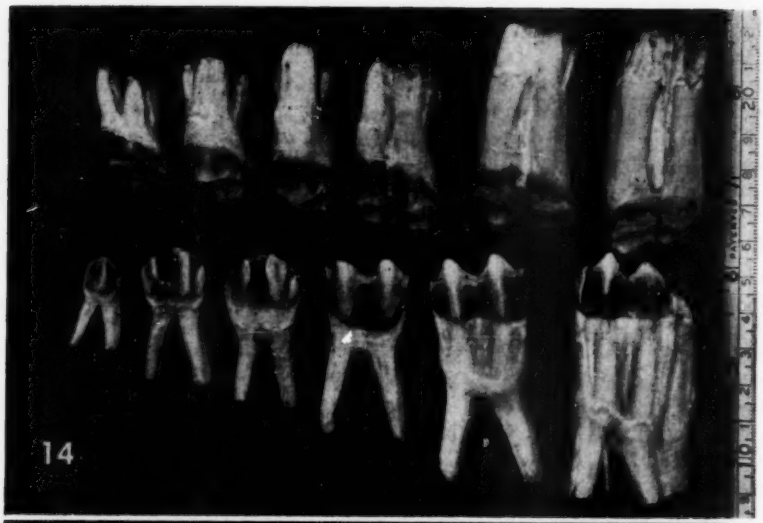


Fig. 14—A medial view of the cheek teeth of a normal 3-year-old Hereford steer. The teeth are arranged in an exaggerated oppositional pattern from which the true pattern may be discerned. Note the accessory roots on third and sixth mandibular cheek teeth. Look closely for the vertical grooves on the maxillary teeth.

Fig. 15—A posterior surface view of the cheek teeth of a normal 3-year-old Hereford steer.

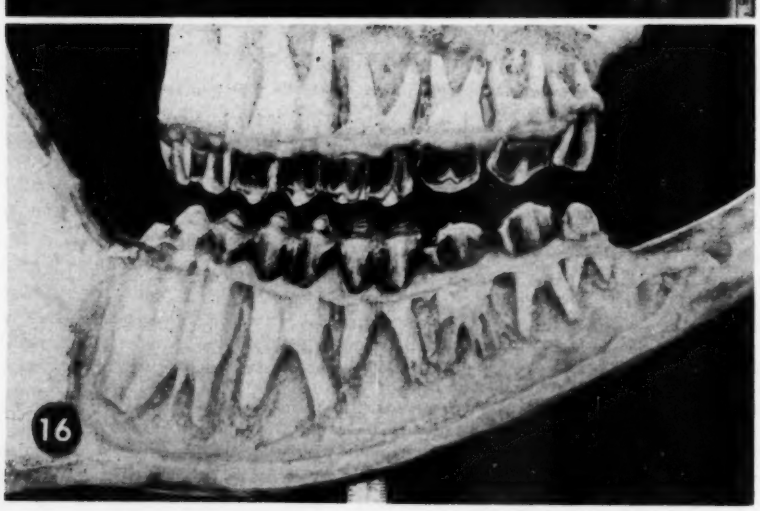


Fig. 16—A lateral dissection of the cheek teeth of a normal 3-year-old Hereford steer. The gingival margins are not removed. The roots of the third mandibular premolar were fractured in dissection.

teeth can not exist when the mouth is closed. Comminution of food depends primarily upon the lateral movements of the mandible.

The arrangement of the mandibular cheek teeth, with relation to their opposing elements in the maxilla, is as follows:

P1)—The first mandibular premolar does not ordinarily oppose its upper counterpart to any great extent and therefore has very little comminutional function.

P2)—The second mandibular premolar is opposed by the greater part of the first maxillary premolar, which curves downward and backward from the gingival margin, and by the anterior one half of the second maxillary premolar.

P3)—The third mandibular premolar is opposed by the posterior one half of the second maxillary premolar, which also curves downward and backward, and by the anterior one third of the third maxillary premolar, which curves downward and backward only slightly. It is significant that the posterior portion of the third mandibular premolar slants away from the tip of the cusp at an angle of approximately forty degrees. Since the direction of the upper third premolar is directed downward and backward, it can be seen that the normal process of attrition will eventually allow the occlusal surface of the upper tooth to be directed entirely toward the fourth mandibular cheek tooth, leaving the third mandibular premolar functioning in part against the anterior margin of its maxillary counterpart (fig. 17). This factor in part accounts for the gradual physiological change in the masticatory table of the cheek teeth from a level plane to that of a shallow "V" as seen in older subjects beginning at any time beyond the age of six.

M1)—The first mandibular molar is opposed by the posterior portion of the third maxillary premolar and the anterior three fifths of the first maxillary molar.

M2)—The second mandibular molar is opposed by the posterior two fifths of the first maxillary molar and the anterior two thirds of the second maxillary molar.

M3)—The third mandibular molar is opposed by the posterior one third of the second maxillary molar and by the entire third maxillary molar.

Physiological Response of Permanent Cheek Teeth to Environment and Attrition.—At first, the masticatory surfaces of the cheek are very rough and the cusps are prominent. A constant tension exists within the alveolus exerting a pressure toward the masticatory table, thus keeping the teeth in close opposition as they wear. This pressure gradually causes the eruption of more and more of the anatomical crown. The intra-alveolar pressure is partially balanced by the functional pressure exerted during mastication, serving as a deterrent to the eruption rate. If for some pathological reason the functional pressure is released from any tooth, that tooth will be seen to arise from the alveolus at a faster rate than those remaining in normal opposition. However, the intra-alveolar pressure decreases as age advances.

As attrition continues, the primary and secondary dentine is soon exposed and the cusps wear rather fast. The tooth has a tendency to level off, with the enamel ridges slightly elevated above the dentinal and cemental portions of the masticatory surface. The deposition of dental calculus on all portions of the teeth, except the masticatory surfaces and highest points of the enamel convolutions on the medial and lateral margins, makes it difficult to appraise the quality of the enamel in the living subject. Only the unusually prominent variations from the normal can be seen.

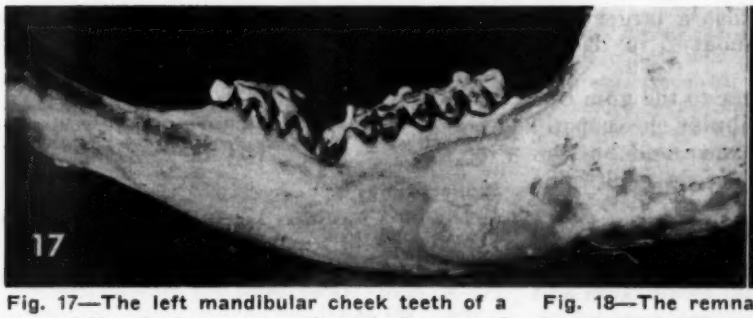


Fig. 17—The left mandibular cheek teeth of a normal Hereford cow approximately 9 years of age. The fourth cheek tooth has nearly worn in two. The alveolar bone has receded at that point. Note the "V" pattern of the masticatory table.

Fig. 18—The remnants of the fourth mandibular cheek tooth removed from a normal aged grade beef-type cow. The tooth has been worn in two, root resorption is well advanced, and heavy irregular layers of cementum cover the persisting portions of the roots.

At a variable age, anywhere from 6 years and beyond (earlier in beef breeds than in dairy), the masticatory table begins to change from level to a shallow "V" with the apex of the "V" at the center of the first mandibular molar which may eventually wear entirely through at the center, leaving only remnants of the anterior and posterior portions (fig. 18). Eleven out of every 14 animals 8 years of age and over examined in abattoirs during this study demonstrated the "V" pattern of wear.

Resorption of the roots of the cheek teeth begins late in their useful life with the result that the tooth may be lost entirely. As the process of resorption takes place, increasingly thick rough irregular layers of cementum are deposited on the root (fig. 18). The pulpal portion may recede entirely with the formation of secondary dentine. The apical foramina may become obliterated, and large masses of cementum cover the apex of each root. Physiologically, the teeth are usually worn out in the order in which they erupt in each jaw, excepting the first mandibular premolar.

Since the first mandibular molar is opposed in part by the third maxillary premolar which formed approximately two years later, it can be readily understood why the wear of the lower tooth is comparatively early and ultimately so complete. Calcifying as they do in the first six months of extra-uterine life during which the animal is facing its most critical period, the first molars are more apt to be of inferior quality, when variation exists, than the rest of the teeth. The third permanent premolar, on the other hand, in addition to being 2 years younger than the mandibular first molar, has a larger anatomical crown which is about ¼ in. deeper from the tip to the root.

The second tooth to wear to the gum line usually is the fifth mandibular cheek tooth. It also may be split by wear and be lost. The variation in wear increases with the age of the animal.

Concurrent with terminal wear and ultimate loss of the cheek teeth, there is a regression of the periodontal membranes and later of the bony alveolar processes which change the shape of the mandible. It then appears to bend at the level of the fourth cheek tooth, running at a greater angle upward and forward from that point (fig. 17).

Gingival Response to Dental Eruption.—Since gingivitis can be caused by a great number of deleterious influences, it will not be discussed in this series of articles, except as later referred to. However, the appearance of the gingiva at time of dental eruption should be described. Since tooth eruption is a process conducive to considerable inflammation and possible local bacterial activity, the gingiva become swollen, and prolifieration of connective tissue is relatively great. As a result, the erupting tooth has a strong tendency to carry the soft tissues along with it. Indeed, incisor teeth have been observed to be in a position of full eruption before breaking through the investing soft tissues. Recession of the inflamed gingiva sometimes requires as much as a year following eruption, and scar tissue may be in prominent evidence. The clinician must use caution in ascribing gingivitis to disease processes until after the physiological gingivitis of dental eruption has subsided.

SUMMARY

That portion of the gross anatomy and physiology of the teeth of the ox, of which knowledge is necessary in dental clinical diagnosis, has been investigated and described. A table for the purpose of approximating the age of mature cattle has been set forth. The changes which take place in the teeth of cattle from birth to old age are described in detail. A brief note on the gingivitis of dental eruption is included. This information, when added to that given in part I of this series of papers, will have provided the clinician with a solid foundation for rational diagnoses of dental irregularities and pathology.

References

[1] Sisson, S. S.: The Anatomy of the Domestic Animals. 2nd ed., W. B. Saunders Co., Philadelphia, (1930): 448.

[2] Huidekoper, R. S. Age of the Domestic Animals. Alexander Eger, Chicago, 1903.

Bibliography

Colyer, F.: Variations and Diseases of the Teeth of Animals. John Bale, Sons and Danielsson, Ltd., London, (1936): 76.

Thoma, K. H.: Oral Pathology. 3rd ed., C. V. Mosby Co., St. Louis, (1950): 286.

Ibid.: 267 to 271.

Ibid.: 271.

Ibid.: 275.

Dorland, W. A. N.: The American Illustrated Medical Dictionary. 22nd ed., W. B. Saunders Co., Philadelphia, (1951): 940.

A Cytological Study of Costal Marrow
II. The Adult Cow

M. LOIS CALHOUN, D.V.M., Ph.D.

East Lansing, Michigan

IN PART I of this article, "The Cytology of the Bone Marrow of the Horse"[1] was presented, including a general literature review of the bone marrow of laboratory and domestic animals, excepting the cow. As in the horse, little research has been done on bone marrow of cattle.

In studies of marrow of domestic animals, both Varićak[2] and Hjärre[3] included the cow. Other studies on bovine marrow include a thesis on bone marrow by Hölzel,*[4] the development of marrow in the metatarsus by Hrestak,*[5] and the work of Marcato[6] on the normal bone marrow. The same year, Marcato[7] made a study of bone marrow in bovine fascioliasis. Mitchell[8] discussed hyperplasia of the bone marrow and osteohematochromatosis in a yearling steer and stated that the bone marrow "exhibited histological changes." No normal picture was given. Stasney and Feldman[9] made hematological and histological studies of the bone marrow from the femur of a calf with leukemic lymphoblastoma but again no normal study was used for comparison. Richter,[10] in discussing leukemia in animals, said that lesions were not found in the bone marrow of cattle. According to Jarmai,[11] histological investigations of the bone marrow of the cow in leukosis were made by Endres,[12] Lengwenat,[13] and Töllner.[14] However, they did not mention that any normal histological studies were made for comparison. Ellenberger[15] gave a brief resumé of the bone marrow in domestic animals with scant reference to any specific animal.

GROSS ANATOMY

Varićak[2] studied the bones of 300 cattle macroscopically and found that the deposition of fatty marrow occurred much earlier in cattle than in horses. This process started in cattle in the axial skeleton as early as 9 months. Varićak explained the few granulocytes and the large number of lymphocytes in bovine blood by the large amount of fat in the bone marrow. He found some of the thoracic vertebrae to be completely filled with fatty marrow. The dorsal end of the ribs retained red marrow, the central part had both red and yellow marrow, and the sternal end became a reservoir for fat. The sternum also contained areas of fatty marrow but with foci of red marrow.

The gross anatomy of the bone marrow was considered in Part I of this study.

MATERIAL AND METHODS

Obtaining the Sample.—The animals used in this investigation consisted of 13 cows and 1 bull in a herd used by the Department of Veterinary Obstetrics at Iowa State College. The same problem existed in getting marrow from the cow as from the horse (Part I). Heavy muscles cover the bovine sternum and it seemed inadvisable to place the animal in a prone position in order to obtain marrow there. As in the horse, the ribs were the chosen site. In figure 1, the general area is outlined and figure 2 illustrates the accessibility of the eleventh, twelfth, and thirteenth rib at that site. When the animal was confined in a stock or restrained against the side of a stall, there was little resistance.

The back and side of the cow should be brushed with a grooming brush and a large area wiped clean with a damp cloth. By palpation, the rib with the least amount of fascia covering it was located. This site was shaved or closely clipped, washed with soap solution, dried, and tincture of iodine was applied. Anesthesia was obtained by injecting about 10 cc. of a 2 per cent procaine hydrochloride solution first into the skin, next into the fascia, and finally on the periosteum. A short incision was then made through the above layers. A No. 487 Goodell-Pratt * hand drill equipped with a straight shank $\frac{3}{32}$-in. jobbers' drill was used to bore into the marrow cavity.

To avoid missing the marrow cavity, care was taken to enter the middle of the rib. When the marrow cavity was entered, the drill tended to "give" a bit. Following removal of the drill, a needle trocar (Jen-Sal No. 39121 **) with the same outside diameter as the drill was inserted, the stilet removed, and an airtight 10-cc. syringe attached to aspirate 1 cc. or less of marrow. The marrow sample was transferred to an oxalate tube and mixed thoroughly by tapping the tube. (*See* Part I for reference to the preparation and efficacy of oxalate tube preparations.) The incision was left open following removal of the cannula but iodine was applied. Healing occurred rapidly and once the hair had grown out, the operative site could not be detected. With the exception of features necessitated by animal variation, the above technique was patterned after that of Osgood and Brownlee[16] for sternal puncture in man.

Taken from a thesis submitted to the Graduate School of Iowa State College, Ames, in partial fulfillment of the requirements for the degree of doctor of philosophy.

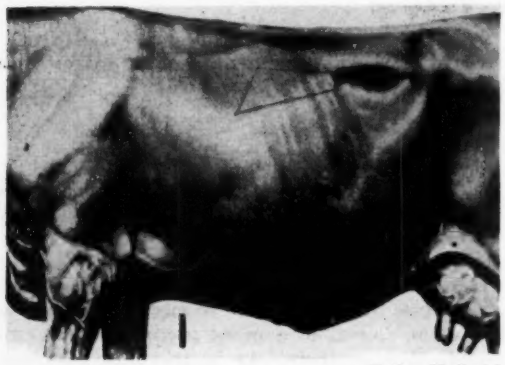
Dr. Calhoun is professor and head, Department of Anatomy, School of Veterinary Medicine, Michigan State College, East Lansing.

*Not available.

* Goodell-Pratt Co., Greenfield, Mass.

** Jen-Sal Laboratories, Kansas City, Mo.

According to Sabin,[17] an analysis of the blood picture is a necessity for understanding the cytology of bone marrow. Blood samples were taken prior to procuring the marrow, as the latter procedure might excite the animal enough to alter the blood picture. The blood was taken from



—*Esther M. Smith*

Fig. 1—Lateral view of the cow showing the general area in which to drill into the ribs for marrow samples.

the jugular vein and oxalated. Conner[18] described this technique. Kainer and Dickson[19] have recently described the anatomy of this vein in relation to venipuncture.

The blood and marrow samples were removed to the laboratory and smears of both were made immediately. Hemoglobin determinations and total red and white counts were done on the blood sample using Osgood's[20] technique. The author found, as did Nordenson[21] and Kandel and LeRoy,[22] that because marrow samples varied so much due to dilution with blood that cell counts had little or no value.

The above technique on the living animal will give the optimum normal cytological picture. Should postmortem examination of the marrow be necessary, Wintrobe[23] preferred the sample be obtained within two hours after death to avoid autolysis. In a study of postmortem changes in human bone marrow, Rohr and Hafter[24] concluded death had little effect on the myeloid elements, but the erythrocytic elements continued to mature. They also found that the neutrophils decreased in number and the nucleus of those remaining became swollen and vacuolated after two hours.

For the staining and counting techniques and for references to the literature in that area, the reader is referred to Part I of this article.

Bone Marrow Studies

Terminology.—The literature review and a discussion of various terms has been presented in Part I.

The terminology used in this paper and the description of these cell types in the cow follow.

Stem cell—all the immature cells which could not be classified into any given series.

Erythroblast—all cells in the red blood cell series from the youngest that could be identified with that series to the normoblast.

Normoblast—those in the red blood cell series that contained hemoglobin in comparable amounts to the adult red blood cells as indicated by similar staining properties and containing nuclei.

Promyelocyte—young cells with nonspecific azurophilic granules.

Eosinophilic and neutrophilic myelocytes—cells in these respective series included the metamyelocytes, juvenile cells, and stab, rod, or band forms of other authors.

Eosinophil and neutrophil—the adult cells of these granulocyte series.

Basophil—there were so few basophils that both developing cells and adults of that series were grouped together.

Lymphocyte—any developing cells in this series were included.

Monocyte—all promonocytes and distinguishable monoblasts were added here.

Plasma cell—if any developing plasma cells were observed, this heading included them.

Myelograms.—Table 1 shows the individual myelograms for the 14 animals used in this experiment.

Table 2 contains a summary of this study compared to data prepared by other authors.

Description of Cells.—Stem Cell.—Its large nucleus exhibited a finely reticulated karyoplasm with two or more pale blue nucleoli and a distinct nuclear membrane. The mottled greyish blue cytoplasm occasionally contained one or two vacuoles and a few azure-staining granules.

—*Esther M. Smith*

Fig. 2—Lateral view of the cow with skin and superficial fascia removed to show what portion of the lateral surface of the ribs is relatively exposed.

TABLE 1—Percentage Distribution of the Marrow Cells from the Ribs of 14 Cows

Cell types	Cow (No.)													Range	Mean
	*42243	42743	5643	52043	52743	61643	61843	62343	62843	7543	7743	71243	72143	72843		
Stem cell	1.4	2.0	3.4	0.8	0.2	0.0	2.2	3.0	2.2	3.2	5.0	2.4	2.8	1.4	0.0 to 5.0	2.14
Erythroblast	15.6	36.2	29.2	20.6	30.2	11.8	42.4	20.0	36.6	37.0	38.6	30.8	42.8	31.8	11.8 to 42.8	30.26
Normoblast	21.4	19.8	18.0	13.6	27.8	9.2	28.6	7.2	20.6	15.4	33.6	22.2	27.0	39.2	7.2 to 39.2	21.69
Total erythroid cells (E)	37.0	56.0	47.2	44.2	58.0	21.0	71.0	27.2	57.2	52.4	72.2	53.0	69.8	71.0	21.0 to 72.2	52.66
Promyelocyte	3.0	1.2	0.6	1.4	0.0	1.8	2.0	6.8	1.6	0.8	0.6	0.6	0.8	0.0	0.0 to 6.8	1.51
Neutrophilic myelocyte	19.8	24.8	16.2	32.0	21.0	28.8	16.4	29.2	17.2	13.0	10.4	15.8	16.2	10.6	10.4 to 32.0	19.39
Neutrophil	9.4	5.0	4.8	4.6	7.2	12.2	1.4	10.0	4.0	8.4	2.4	6.2	1.2	3.4	1.2 to 12.2	5.73
Eosinophilic myelocyte	4.6	5.6	9.2	7.8	3.0	9.0	2.8	9.8	9.0	10.4	6.0	8.4	6.2	1.8	1.8 to 10.4	6.69
Eosinophil	7.6	0.0	1.0	0.6	1.0	2.4	0.0	4.2	0.2	3.0	0.2	3.0	0.2	3.6	0.0 to 7.6	1.92
Basophils (all)	0.2	0.0	0.4	1.0	0.8	0.2	0.4	0.4	0.8	0.0	0.0	0.4	0.0	0.2	0.0 to 1.0	0.34
Total myeloid cells (M)	44.6	36.6	32.2	47.4	33.0	54.4	23.0	60.4	32.8	35.6	19.6	34.4	24.6	19.6	19.6 to 60.4	35.59
Monocyte	0.0	1.2	5.6	5.2	1.2	7.6	1.4	1.6	3.4	2.2	1.2	4.4	0.2	1.8	0.0 to 7.6	2.64
Plasma cell	0.8	0.8	0.4	2.0	1.0	0.2	0.4	1.4	0.8	1.0	0.6	1.0	0.2	0.4	0.2 to 2.0	0.79
Lymphocyte	17.2	3.4	11.2	10.4	6.6	16.8	2.0	6.4	3.6	5.6	1.4	4.8	2.4	5.8	1.4 to 17.2	6.68
Megakaryocytes in 300 sq. mm.	21	41	23	20	0	8	0	121	7	8	5	8	83	7	0 to 121	25.14
Mitoses	3	7	2	4	5	2	5	0	4	9	9	5	11	3	0 to 11	4.9
Myeloid-erythroid ratio	1.2	0.65	0.68	1.07	0.57	2.59	0.32	2.22	0.57	0.68	0.27	0.65	0.35	0.28	0.27 to 2.59	0.676

* All female except 72843.

Erythroblasts.—This classification included any cell from the earliest that could be differentiated from a stem cell to and including those with considerable hemoglobin but not yet having the cytoplasmic staining qualities of the adult red blood cell. The youngest nuclei stained a homogeneous reddish purple color and contained nucleoli. As the cells matured the nuclei became smaller, lost their nucleoli, and stained more densely due to the clumped chromatin. The structureless cytoplasm changed in color from blue to grey with a tinge of green apparent as the hemoglobinization became more extensive.

Normoblast.—In this cell, hemoglobinization had been completed and the cytoplasm stained like the adult red blood cell but still retained a small pyknotic nucleus. As in the horse, considerable variation in development was apparent, some cells exhibited mature cytoplasm with a young nucleus while others were just the reverse.

Promyelocyte.—It presented a nucleolar-containing spherical or ovoid reddish purple nucleus in a light blue cytoplasm containing many rather small azurophilic granules. The cells varied in size from 15 to 22 μ in diameter.

Neutrophilic Myelocyte. — All developmental stages of the neutrophil series were grouped under this heading. They varied in size from 10 to 17 μ in diameter. The youngest nucleus was spherical, with fine chromatin material. As it developed the nucleus became oval, kidney-bean shaped, u-shaped, and finally constricted with a concomitant darker staining of the nucleus due to the clumping of the chromatin. The earliest stages often retained some of the azurophilic granules of the promyelocyte in addition to the neutrophilic granules in the pale blue cytoplasm. These azurophilic granules disappeared as the cell matured.

Eosinophilic Myelocyte.—The developmental stages were similar to those of the neutrophil. They were readily identified by the eosinophilic granules which were about 1 μ in diameter. The small size of the granules, and their density in the cytoplasm precluded any accurate counting. The largest cells observed in the cow measured 26 by 26 and 22 by 30 μ. Considerable variation in color of the granules, from azurophil to a bluish grey, was

noted in the younger cells. The granules quite frequently obscured the blue-grey cytoplasm. The nucleus went through a succession of shapes like the nucleus of the neutrophil from spherical to the bilobed nucleus of the adult.

Basophils.—The largest cell was about 20 μ in diameter. Its reddish purple nucleus was most usually obscured by the more deeply stained basophilic granules which filled the cell. These granules measured about 1 μ.

Plasma Cell.—The eccentrically placed nucleus with its perinuclear clear area usually distinguished this cell from similarly stained cells in the lymphocytic and erythrocytic series. Furthermore, the cytoplasm tended to exhibit a special blue color. As in other series, the younger the cell the less dense the chromatin network.

Megakaryocyte. — This was the largest cell in bone marrow. They were large, spherical, or ovoid cells often with pseudopodia. They varied in size from 20 to about 100 μ. Two distinct cytological pictures were observed. The first exhibited two types of cells. One had a dark plum-colored nucleus with an area of reddish purple granular cytoplasm. At the outer edge of the cytoplasm, the granules were absent or sparse and the blue cytoplasm was apparent (fig. 10). The second presented the exact opposite picture, a perinuclear area of blue and an outer rim of fine reddish purple granules. The other cytological picture presented a cell with a less dense, lobed or folded nucleus surrounded by a cytoplasm filled to the edge with tiny reddish purple granules. In some cells, the cytoplasm appeared to fray out into particles resembling platelets (fig. 9).

SAMPLING EXPERIMENT

A sampling experiment consisted of getting marrow from two positions about an inch apart on each of five ribs in the cow. The ribs used were the eighth to twelfth and the body level was that indicated in figure 2. These data are shown in table 3.

The means shown in table 4 were treated statistically by analysis of variance. The results showed three significant differences in the twelfth rib and no significant difference between positions on the rib. The total erythroid cells indicated a significant negative trend from the eighth to the twelfth rib and the neutrophils a significant positive trend in the same rib order. Since there was only 1 animal and many of the cell types occurred in such few numbers, the experiment should not be given

TABLE 2—Comparison of Cow Data with Other Myelograms

Marcato [1]	Middle-aged cow	Old cows	Mean	Hjärre [2]	Range	Author	Range from table 1	Mean from table 1
Hemocytoblast	4.35	4.1	4.23	Myeloblast	1.5 to 4.0	Stem cell	0.0 to 5.0	2.14
Erythroblast	3.7	3.68	3.69					
Proerythroblast	2.3	2.42	2.36	Pronormoblast	0.0 to 1.0			
Basophilic erythroblast	11.5	11.95	11.72	Basophilic				
Total erythroblasts	17.5	18.05	17.77	normoblast	3.0 to 7.5	Erythroblast	11.8 to 42.8	30.26
Polychromatophilic erythroblast	18.0	16.8	17.4	Hemoglobin				
Orthochromatic erythroblast	19.5	19.0	19.25	containing				
Total	37.5	35.8	36.65	normoblast	27.0 to 55.0	Normoblast	7.2 to 39.2	21.69
Total erythroid series	55.0	53.85	54.42			Total erythroid cells	21.0 to 72.2	52.66
Neutrophilic promyelocyte	1.4	1.5	1.45	Promyelocyte	0.5 to 3.0	Promyelocyte	0.0 to 6.8	1.51
Neutrophilic myelocyte	5.6	6.1	5.85	Myelocyte	3.0 to 9.6			
Neutrophilic metamyelocyte	11.5	12.1	11.8	Metamyelocyte	3.0 to 13.5	Neutrophilic		
Total neutrophil series	18.5	19.7	19.10	Stab cells	7.6 to 18.1	myelocyte	10.4 to 32.0	19.39
				Segmented neutrophil	6.3 to 19.0	Neutrophil	1.2 to 12.2	5.73
Eosinophilic promyelocyte	1.5	1.5	1.5			Eosinophilic		
Eosinophilic myelocyte	5.0	5.0	5.0			myelocyte	1.8 to 10.4	6.69
Eosinophilic metamyelocyte	6.3	5.27	5.78			Eosinophil	0.0 to 7.6	1.92
Total eosinophil series	12.8	11.77	12.28			All basophils	0.0 to 1.0	0.34
						Total myeloid cells	19.6 to 60.4	35.59
Monoblast and monocyte	8.35	9.36	8.85	Monocyte	0.0 to 1.0	Monocyte	0.0 to 7.6	2.64
Plasmacyte	0.5	0.68	0.59			Plasma cell	0.2 to 2.0	0.79
						Lymphocyte	1.4 to 17.2	6.68
Myeloid-erythroid ratio	0.58	0.66	0.62	M.E. ratio	0.7	M/E	0.27 to 2.59	0.676

TABLE 3—Cellular Composition of Marrow in Various Ribs and Upper and Lower Positions on Those Ribs of the Same Cow

Rib →	8 u.	8 l.	9 u.	9 l.	10 u.	10 l.	11 u.	11 l.	12 u.	12 l.
Stem cell	1.0	0.8	0.2	0.2	0.8	0.4	0.8	1.0	0.6	0.0
Erythroblast	20.0	16.0	14.4	8.8	20.0	12.4	6.8	13.0	4.2	3.4
Normoblast	19.0	18.0	19.8	21.2	17.2	10.0	8.2	15.4	18.2	3.6
Total erythroid cells (E)	39.0	34.0	34.2	30.0	37.2	22.0	15.0	28.4	22.4	7.0
Promyelocyte	2.0	2.0	1.6	0.6	1.2	0.8	1.6	1.0	0.4	0.3
Neutrophilic myelocyte	23.2	26.0	28.0	30.6	22.8	25.0	38.0	34.4	26.8	18.5
Neutrophil	3.6	2.4	2.6	7.6	4.4	11.6	11.0	7.6	10.4	14.5
Eosinophilic myelocyte	8.4	12.0	13.0	10.2	9.8	12.6	17.0	13.0	10.2	10.1
Eosinophil	2.6	3.4	2.8	2.4	1.8	4.2	3.0	10.0	6.0	3.0
Basophils (all)	1.0	0.4	0.0	0.0	0.4	0.0	0.6	0.2	0.4	0.4
Total myeloid cells (M)	40.8	46.2	48.0	51.4	40.4	54.2	71.2	66.2	54.2	46.8
Monocyte	2.2	2.4	7.2	3.4	3.2	3.6	1.6	5.0	0.8	7.1
Plasma cell	1.0	0.0	0.4	0.0	1.2	0.4	1.0	0.2	0.0	0.0
Lymphocyte	16.0	16.6	10.0	15.0	17.2	19.0	10.4	8.2	22.0	39.1
Myeloid-erythroid ratio M/E	1.04	1.36	1.40	1.71	1.08	2.46	4.74	2.29	2.41	6.68

u.—upper; l.—lower.

too much weight until further work can be done to substantiate it. In the light of these meager data, it would seem better to obtain samples anterior to the twelfth rib. In view of Varićak's [2] findings, that the sternal end of the rib changed from red to yellow marrow earlier than the vertebral end, probably it is wise to drill the rib at as high a level as possible and still avoid the back muscles.

DISCUSSION

In the last two decades, great strides have been made in human medicine in correlating the bone marrow findings and certain diseases. Some of those investigations are mentioned here that they may point the way to similar researches in the field of veterinary hematology.

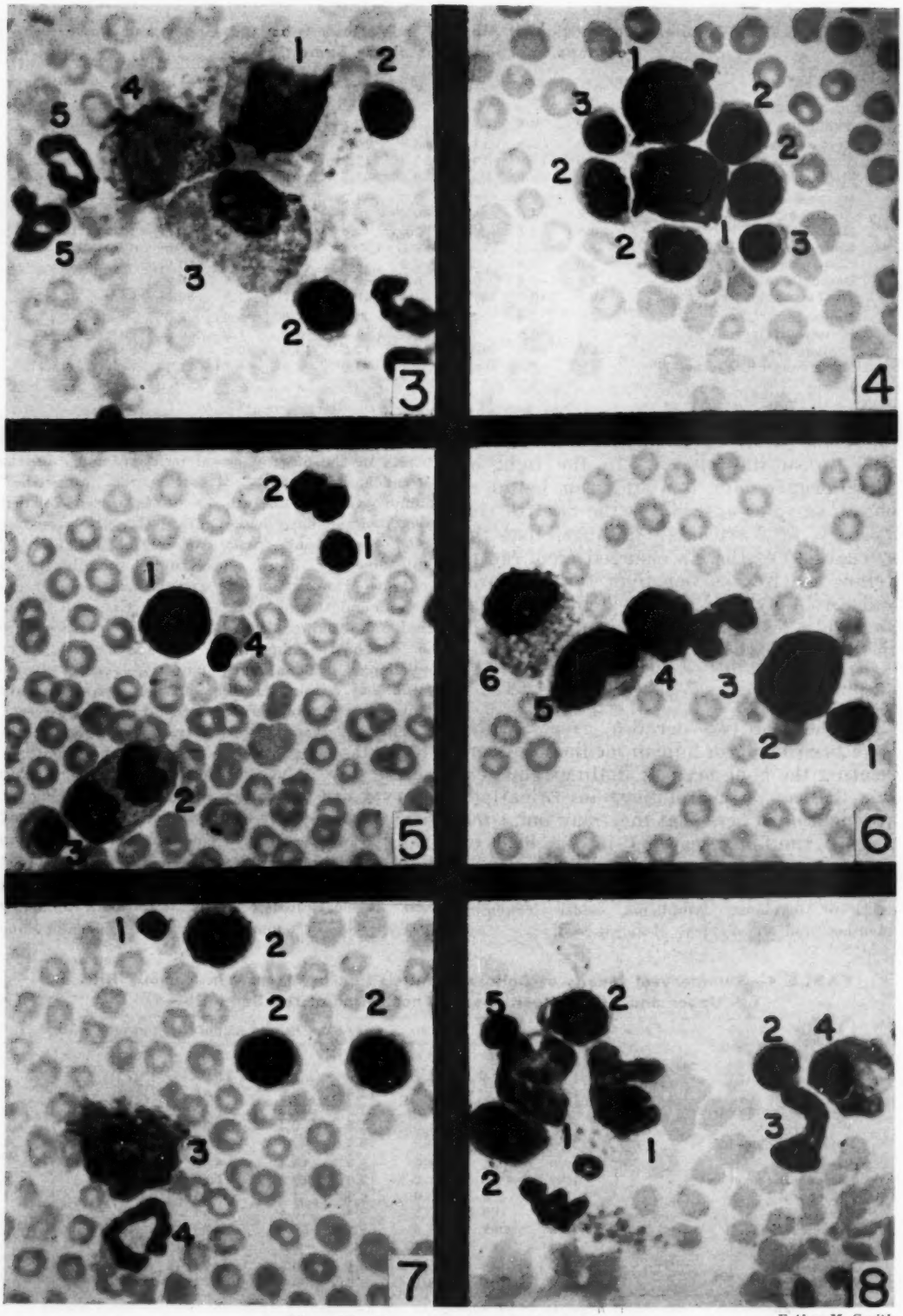
Dreyfus [25] valued bone marrow examination in cases of myeloma, lymphoma, acute leukemia, anemias, and in atypical Hodgkin's disease. According to van de Merwe,[26] the absence of megaloblasts in the marrow ruled out carcinoma of the stomach. Dameshek, Henstell, and Valentine [27] found sternal puncture to be indicated in persistent anemia, leukopenia, thrombocytopenia, splenectomy, and for a differential diagnosis of splenomegaly. Jagić and Klima [28] thought that a marrow examination was useful in diseases of the reticuloendothelial system. Vogel, Erf, and Rosenthal [29] determined that bone marrow studies were of diagnostic importance in Gaucher's disease, myeloma, leishmaniasis, malaria, certain leukemias and carcinomas as well as of confirmatory value in a number of other blood dyscrasias.

Zanaty [30] thought some workers exaggerated the diagnostic value of bone marrow counts but admitted their value in the study of the morbid anatomy and physiology of the blood-forming organs and the effects of treatment. Hardgrove and Van Hecke [31] resorted to bone marrow examination in questionable blood dyscrasias and certain blood disorders. Hynes [32] observed that aplastic anemia and myelosclerosis necessitated bone marrow study. Schmid [33] made a study of blood and bone

TABLE 4—Summary of Means of Cellular Composition of Marrow in Various Ribs and Upper and Lower Positions on Those Ribs of the Same Cow

Rib →	8	9	10	11	12	Upper	Lower	Total
Stem cell	0.9	0.2	0.6	0.9	0.3	0.68	0.48	0.58
Erythroblast	18.0	11.6	16.2	9.9	3.8*	13.08	10.72	11.90
Normoblast	18.5	20.5	13.6	11.8	10.9	16.48	13.64	15.06
Total erythroid cells**	36.5	32.1	29.6	21.7	14.7	29.56	24.28	26.92
Promyelocyte	2.0	1.1	1.0	1.3	0.4*	1.36	0.94	1.15
Neutrophilic myelocyte	24.6	29.3	23.9	26.2	22.6	27.76	26.90	27.33
Neutrophil†	3.0	5.1	8.0	9.3	12.4	6.40	8.74	7.57
Eosinophilic myelocyte	10.2	11.6	11.2	15.0	10.2	11.68	11.58	11.63
Eosinophil	3.0	2.6	3.0	6.5	4.5	3.24	4.60	3.92
Basophil	0.7	0.0	0.2	0.4	0.4	0.48	0.20	0.34
Total myeloid cells	43.5	49.7	47.3	68.7	50.5	50.90	52.96	51.93
Monocyte	2.3	5.3	3.4	3.3	3.9	3.0	4.3	3.65
Plasma cell	0.5	0.2	0.8	0.6	0.0	0.72	0.12	0.42
Lymphocyte	16.3	12.5	18.1	9.3	30.6*	15.12	19.58	17.35

* Significant; ** significant negative trend among the rib averages; † significant positive trend among the rib averages.



Magnification approximately x 950.

—*Esther M. Smith*

marrow in human undulant fever. He observed "Bang nodules" in the marrow and thought the platelet-forming function of the megakaryocytes was interfered with. Vogel and Bassen [34] used sternal puncture to rule out leukemia in cases of infectious mononucleosis. Jones [35] studied the bone marrow in hyperthyroidism and hypothyroidism and found an increase in nucleated cells in the former and a decrease in the latter. Limarzi and Schleicher [36] worked on the reaction of peripheral blood and bone marrow in chronic hemorrhage and essential thrombopenic purpura and concluded that diagnoses of purpuric states could be more satisfactorily made form bone marrow studies. Davidson [37] confirmed diagnoses of cancer, Hodgkin's disease, multiple myelomatosis, and lipoidoses by finding specific cellular elements in the bone marrow. Gordon [38] summarized the value of bone marrow studies under four headings: to aid in the diagnosis of obscure conditions, to confirm diagnoses, to further the understanding of disease processes, and as a method of bio-assay of certain drugs.

Falconer and Leonard [39] used marrow technique as a means of gaining additional information and suggested its interpretation in the light of other data. They recommended brucellosis and bacterial endocarditis as a field for further study. Beizer, Hall, and Giffin [40] confirmed suspected myeloma by finding myeloma cells in the bone marrow. Dameshek [41] considered bone marrow biopsy an important tool in the differential diagnosis of refractory anemia and pancytopenia. He also ruled out leukemia in cases of thrombopenic purpura and hemolytic anemia by this method. Kienle [42] distinguished erythroblastosis and erythroleukemia by bone marrow studies. In addition to those hemopoietic disorders already mentioned, Turkel and Bethell [43] claimed marrow aspiration from the sternum to be an almost universal practice in disturbances of the reticuloendothelial system, certain infectious diseases, and neoplasms of the marrow cavity.

Williams [44] found the megakaryocyte count in primary pneumonia to be more than 5,000 cells per cubic millimeter and suggested a definite relationship between pneumonia and hyperplasia of megakaryocytes.

Bloom [45] is pioneering in the veterinary field in studying bone marrow in various diseased conditions in the dog. Some of his findings may be noted here: increased myeloid-erythroid ratio in pyometra and aregenerative anemia; neutrophilic hyperplasia in marrow in pyometra and streptothrichosis; decreased myeloid-erythroid ratio and hyperplasia of the erythroid series in advanced filariasis; and the occurrence of lymphoma cells in the bone marrow in malignant lymphoma.

If future bone marrow studies should lead to an earlier diagnosis of obscure blood dyscrasias in our farm animals, it would be of considerable economic value. Mitchell [46] suggested that an early diagnosis of lymphocytoma in cattle would result in a saving in feed and care.

In conclusion, the fact may be emphasized that this is only a fragment of the studies that could and need to be made. Further work may reveal a more refined technique of obtaining marrow from these animals. Schleicher [47] has completed a study on the volumetric pattern of sternal marrow in man, including the fat, plasma, myeloid-erythroid ratio, and erythrocyte content per cubic centimeter. Such a study would be valuable in animals. Wirth [48] investigated the reactions of the hematopoietic system of the various domestic animals in response to bleeding. He found that it varied with the species. Could this variation have been due to inherent differences in the bone marrow? No bone marrow studies were made. The gross changes associated with age and variations with sex are yet to be worked out for the various species of animals. Are lymph nodules present in the marrow of any of our domestic animals? There is a difference of opinion regarding their presence in man. Jaffé [49] gave the weight of human marrow in percentage of the body weight, 3.4 to 5.9, and found 5% Gm. of marrow per gram of blood. He further stated that half of the bone marrow of an adult person is ac-

Legends for Illustrations on Opposite Page

Fig. 3—1. Stem cell; 2. erythroblasts; 3. eosinophilic myelocyte; 4. eosinophilic metamyelocyte; 5. adult neutrophils.

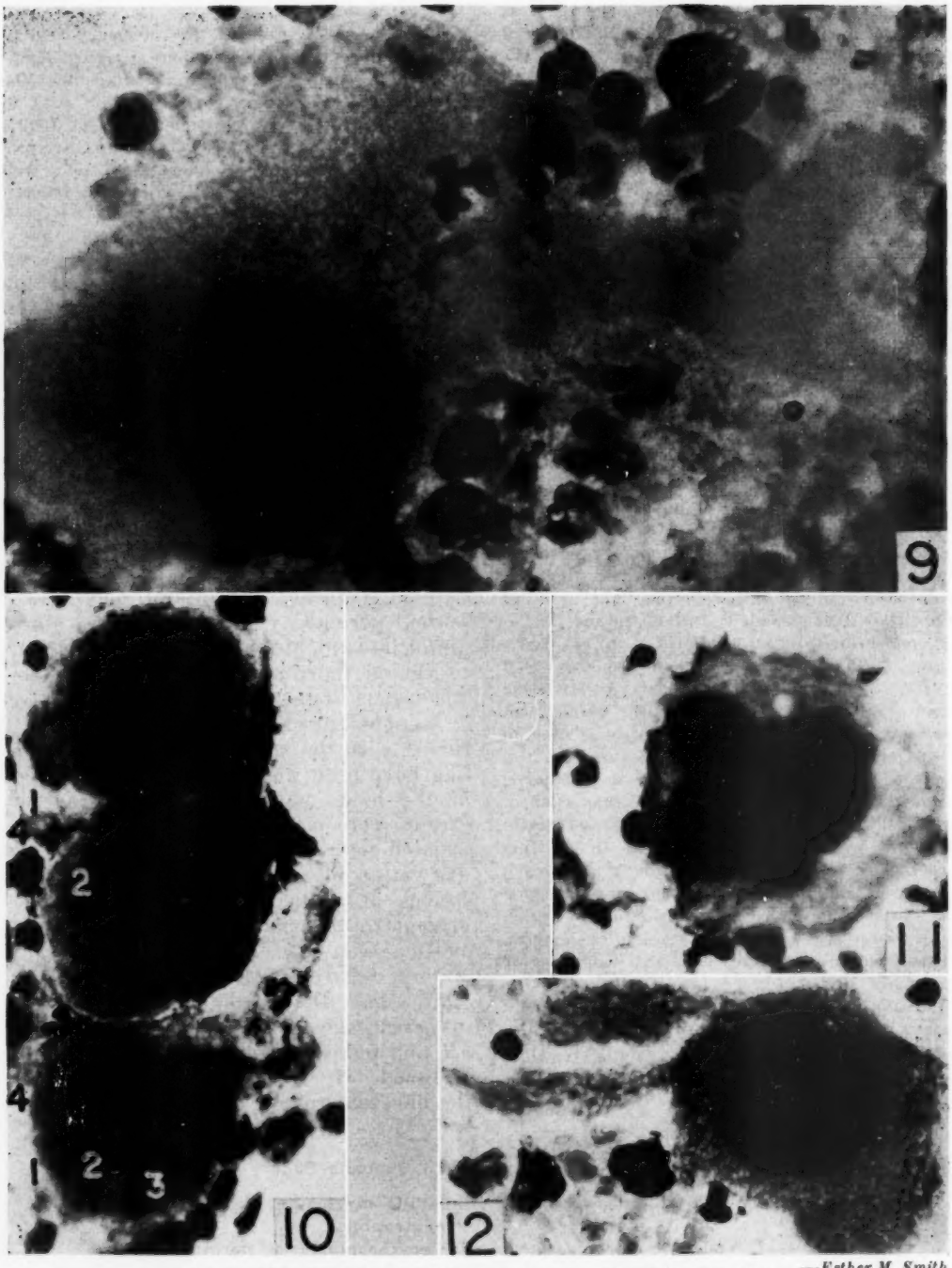
Fig. 4—1. Young erythroblasts; 2. older erythroblasts; 3. normoblasts.

Fig. 5—1 Erythroblasts; 2. erythroblasts in mitosis; 3. normoblast; 4. normoblast shedding its nucleus.

Fig. 6—1. Normoblast; 2. stem cell; 3. neutrophilic band cell; 4. erythroblast; 5. neutrophilic metamyelocyte; 6. eosinophilic myelocyte.

Fig. 7—1. Normoblast shedding its nucleus; 2. erythroblasts; 3. basophilic metamyelocyte; 4. neutrophilic band cell.

Fig. 8—1. Monocytes; 2. erythroblasts; 3. neutrophilic metamyelocyte; 4. plasma cell; 5. normoblast nucleus.



—*Esther M. Smith*

Fig. 9—Megakaryocyte with pseudopods (56 x 100 μ). x 1400.
This cell had a blue-staining center with an outer rim of fine azure granules.
Fig. 10—Megakaryocytes (50 x 60 μ). x 760.
1) Pale blue stained outer rim of cytoplasm; (2) denser staining purple central mass; (3) only apparent nuclear mass in the group; (4) fragmenting edge.
Fig. 11—Megakaryocyte (50 x 55 μ). x 800.
Note the ragged edge with fragments (platelets) along the border.
Fig. 12—Megakaryocyte with pseudopods (52 x 115 μ). x 800.

tive and concluded that the weight of the red marrow equaled that of the liver. How would such figures for domestic animals compare? The effect of drugs upon bone marrow of our domestic animals is yet to be determined. In the future, bone marrow may become as important a diagnostic agent in the field of veterinary medicine as it now is in the realm of human medicine.

SUMMARY

The normal cytological picture of the bone marrow of the cow has been investigated and the results are shown in table 1. Samples were obtained from 14 cattle using the ribs as the site for procuring the marrow.

A sampling study was made at two different levels on each of five ribs (8th to 12th) in 1 cow and the cell counts recorded. An analysis of variance of the means showed a significant variation from the mean for the erythroblasts, promyelocytes, and lymphocytes in the twelfth rib. A significant positive trend was observed from the eighth to twelfth rib in the total erythroid cells, and the neutrophils showed a negative trend in the same direction. Since only 1 animal was used, more work along the same line is needed to confirm these data.

In establishing a normal myelogram for the cow, 500 cells were counted for the differential count on the marrow smears. The mitotic figures encountered per 500 cells were recorded. The megakaryocytes were enumerated in a 300-sq.-mm. area. The myeloid-erythroid ratio was determined.

A detailed morphological study was made of individual cells and the "Munsell Book of Color" [50] was used as a reference for color comparisons.

References

[1] Calhoun, M. Lois: A Cytological Study of Costal Marrow. I. The Adult Horse. Am. J. Vet. Res., *15*, (1954): 181-196.

[2] Varićak, T. D.: Zur Kenntnis des Markes der Rumpfknochen. Untersuchungen zwecks klinischer Auswertung an Pferd, Rind, Schwein, Hund und Katze. Arch. f. wissensch. prakt. Tierheilk., *73*, (1935): 461-475.

[3] Hjärre, A.: Om sternalpunktion och den normala benmargsbilden hos husdjuren. Skand. Vet.-tidskr., *33*, (1943): 457-472.

[4] Hölzel, E.: Ueber Knochenmarkspunktion beim Rind. Inaug. Dissertation, Berlin, 1939. Reviewed by Hjärre in Om Sternalpunktion och den normala benmargsbilden hos husdjuren. Skand. Vet.-tidskr., *33*, (1943): 457-472.

[5] Hrestak, M.: Development of Bone Marrow in Bovine Metatarsus. Vet. Arhiv., *11*, (1941): 329-342. Original not seen; Abstract in Wien. tierärztl. Monatschr., *29*, (1942): 379-380.

[6] Marcato, Arnaldo: Il midollo osseo normale nella specie bovina. (Mielogramma, rapporto leucoeritropoietico, curve di maturazione). Nuova vet., *19*, (1941): 173-179. Original not seen; Abstract in Jahresb. Vet.-Med., *69*, (1942): 530.

[7] Marcato, Arnaldo: Bone-Marrow in Bovine Fascioliasis. Nuova Vet., *20*, (1941): 219-223.

[8] Mitchell, G. E.: Hyperplasia of Bone Marrow and Osteohematochromatosis in a Yearling Steer. J.A.V.M.A., *96*, (1940): 741.

[9] Stasney, J., and Feldman, W. H.: Leukemic Lymphoblastoma in a Calf; A Hematologic and Histologic Study. Am. J. Cancer, *34*, (1938): 240-247.

[10] Richter, M. N.: Leucemia. In "Handbook of Hematology" ed. by Hal Downey, *4*, (1938): 2885-3035.

[11] Jarmai, K.: Die Leukosen der Haustiere. Ergebn. d. allg. Path. u. path. Anat., *28*, (1934): 227-312.

[12] Endres, P.: Ein Beitrag zur Kenntnis der lymphatischen Leukämia des Rindes. Wien. tierärztl. Monatschr., *9*, (1922): 107-118.

[13] Lengwenat, H.: Zwei Fälle von leukämischer Lymphadenose beim Rinde. Inaug. Diss., Hannover, 1931.

[14] Töllner, A.: Beitrag zur vergleichenden pathologischen Anatomie der Lymphadenose und der Lymphosarkomatose des Rindes. Inaug. Diss., Hannover, 1931.

[15] Ellenberger, W.: Lehrbuch der Histologie und vergleichenden mikroskopischen Anatomie der Haussäugetiere, sechste neubearbeitete Auflage von A. Trautmann und J. Fiebiger, Paul Parey, Berlin (1931): 114-115.

[16] Osgood, E. E., and Brownlee, Inez E.: Culture of Human Marrow. Details of a Simple Method. J. Am. M. A., *108*, (1937): 1793-1796.

[17] Sabin, F. R.: Bone Marrow. Physiol. Rev., *8*, (1928): 191-244.

[18] Conner, G. H.: Prognostic Value of Blood Determinations in Certain Surgical Conditions of Horses. Am. J. Vet. Res., *6*, (1945): 45-53.

[19] Kainer, R. A., and Dickson, W. M.: An Examination of the Valves of the External Jugular Veins of the Ox with Reference to the Selection of a Site for Venipuncture. J.A.V.M.A., *123*, (1953): 523-526.

[20] Osgood, E. E.: A Textbook of Laboratory Diagnosis. 3rd ed. The Blakiston Co., Philadelphia, 1940.

[21] Nordenson, N. G.: Studies on Bone Marrow from Sternal Puncture. Börtzells, Esselte, Stockholm, 1935.

[22] Kandel, E. V., and LeRoy, G. V.: Limitations of Biopsy of Sternal Marrow. Arch. Int. Med., *64*, (1939): 121-136.

[23] Wintrobe, M. M.: Clinical Hematology. Lea and Febiger, Philadelphia, 1942.

[24] Rohr, K., and Hafter, E.: Untersuchungen über postmortale Veränderungen des menschlichen Knochenmarks. Folia Haemat., *58*, (1937): 38-50.

[25] Dreyfus, A.: Puncture of the Sternum as a Diagnostic Method. J. Am. M. A., *107*, (1936): 365.

[26] Merwe, van de, C. F.: Bone Marrow Studies in the Clinic. Folia Haemat., *55*, (1936): 108-114, 218-226.

[27] Dameshek, W., Henstell, H., and Valentine, E.: The Comparative Value and the Limitations of the Trephine and Puncture Methods for Biopsy of the Sternal Marrow. Ann. Int. Med., *11*, (1937): 801-818.

[28] Jagić, N., and Klima, R.: Ueber die diagnostische Bedeutung der Knochenmarkpunktion. Wien. klin. Wchnschr., *50*, (1937): 363-367.

[29] Vogel, P., Erf, L. A., and Rosenthal, N.: Hematologic Observations on Bone Marrow Obtained by Sternal Puncture. Am. J. Clin. Path., *7*, (1937): 436-447, 498-515.

[30] Zanaty, A. F.: Examination of the Bone Marrow by Sternal Puncture. Lancet, London, *233*, (1937): 958-962.

[31] Hardgrove, M., and Van Hecke, L. J.: Sternal Marrow Aspiration. Wisconsin M. J., *38*, (1939): 111-114.

[32] Hynes, M.: Sternal Puncture. Lancet, London, *236*, (1939): 1373-1379.

[33] Schmid, N.: Blut und Knochenmark bei Morbus Bang. Schweiz. med. Wchnschr., *69*, (1939): 191-193.

[34] Vogel, P., and Bassen, F. A.: Sternal Marrow in Children in Normal and Pathologic States. Am. J. Dis. Child., *57*, (1939): 245-268.

[35] Jones, R. M.: Human Sternal Bone Marrow in Hyperthyroid and Myxedematous States. Am. J. M. Sci., *200*, (1940): 211-220.

[36] Limarzi, L. R., and Schleicher, E. M.: The Reaction of Peripheral Blood and Bone Marrow in Chronic Hemorrhage and in Essential Thrombopenic Purpura. J. Am. M. A., *114*, (1940): 12-18.

[37] Davidson, L. S. P.: Biopsy of the Sternal Bone Marrow as a Diagnostic Procedure. Edinburgh M. J., *48*, (1941): 678-687.

[38] Gordon, H.: A Method for Preparing Smears and Sections of Aspirated Sternal Marrow. J. Lab. and Clin. Med., *26*, (1941): 1784-1788.

[39] Falconer, E. H., and Leonard, M. E.: The Value of Sternal Marrow Aspiration as a Method of Bone Marrow Biopsy. Ann. Int. Med., *15*, (1941): 446-458.

[40] Beizer, L. H., Hall, B. E., and Giffin, H. Z.: The Diagnosis of Multiple Myeloma by Sternal Aspiration. Am. J. M. Sci., *203*, (1942): 829-836.

[41] Dameshek, W.: Hematology: Diseases Other Than Anemia. Bone Marrow. New England J. Med., *226*, (1942): 383-391.

[42] Kienle, F.: Die Leistungsfähigkeit der Sternalpunktion in der Differentialdiagnose von Erythroblastämien. Med. Klin., *38*, (1942): 101-104.

[43] Turkel, H., and Bethell, F. H.: Biopsy of Bone Marrow Performed by a New and Simple Instrument. J. Lab. and Clin. Med., *28*, (1943): 1246-1251.

[44] Williams, R. J.: Hyperplasia of Megakaryocytes in Pneumonia and Its Relationship to Leukoblastic Hyperplasia of Bone Marrow. Am. J. Path., *18*, (1943): 1105-1127.

[45] Bloom, F.: Bone Marrow Biopsies in Normal and Diseased Dogs—Differential Counts. J.A.V.M.A., *107*, (1945): 220-225.

[46] Mitchell, K. P.: Lymphocytosis in a Cow. Vet. Med., *38*, (1943): 493.

[47] Schleicher, E. M.: The Volumetric Pattern of Aspirated Normal Human Sternal Marrow of Males 18 to 40 Years. Am. J. Clin. Path., *14*, (1944): 370-379.

[48] Wirth, D.: Die besondere Reaktionsweise der hämatopoetischen Organsysteme bei unseren Haussäugetierarten. Thirteenth Internat. Vet. Cong., *1*, (1938): 273-281.

[49] Jaffé, R. H.: The Bone Marrow. J. Am. M. A., *107*, (1936): 124-129.

[50] Munsell, A. H.: Munsell Book of Color. Munsell Color Co., Inc., Baltimore, Md., 1929.

"Deperitonealization" of the Wall of the Rumen

EDGAR F. EBERT, D.V.M.; BENJAMIN B. ROSEBOOM, M.S.;
HOMER E. DALE, D.V.M., Ph.D.

Columbia, Missouri

REPEATED penetration of the rumen by tubular instruments (*i.e.* needles, trocars, pipettes) is often complicated by contamination of the peritoneal cavity with ruminal contents. The possibility of this contamination and of consequent peritoneal infection is much greater if the instrument is to be left in place for several hours or longer. The usual rumen fistula circumvents the danger of peritonitis but is usually accompanied by the loss of gas, fluid, or heat; the normality of rumen digestion under such conditions is open to serious question. The procedure described below was designed to "deperitonealize" a portion of the rumen by the formation of adhesions, so that repeated penetration could be accomplished without the danger of peritonitis and without a permanent opening into the rumen.

PROCEDURE

The subject, a 3-year-old Jersey cow, was restrained in the standing position, and the left paralumbar fossa was prepared for surgery in the usual fashion. Paravertebral anesthesia was accomplished by injecting 4 per cent procaine around the last thoracic and first three lumbar nerves. An 8-in. vertical incision was made through the skin, fascia, and external oblique muscle. This incision was started 2 in. below the transverse processes of the lumbar vertebra and was located midway between the tuber coxa and the last rib. The fibers of the internal oblique muscle were separated by blunt dissection, and the transversus muscle and parietal peritoneum were cut so that this wound corresponded in length and direction to the skin incision.

Three inches to each side of the operative incision the parietal peritoneum was scraped with the dulled blade of a cartilage knife until a mild hyperemia was apparent; the corresponding area of visceral peritoneum on the wall of the rumen was scraped in the same fashion. At each of the four corners of the scarified area, a single interrupted suture was placed through the entire thickness of the abdominal wall and through the peritoneum, muscle, and submucosa of the wall of the rumen. The "bite" of these sutures was comparatively large, since their function was to hold the wall of the rumen against the parietal peritoneum until a firm union had formed.

The edges of the parietal peritoneum and transversus abdominis were brought into apposition with a row of continuous catgut sutures; the edges of the skin and external oblique muscle were united with a row of interrupted "figure 8" nylon sutures. Moist heat was applied to the area for several hours on each of the first three postoperative days. The figure 8 sutures were removed on the seventh postoperative day, and the four sutures holding the rumen against the abdominal wall were removed on the fourteenth postoperative day.

RESULTS

The extent of the adhesions was determined by rectal palpation four weeks after the surgery; this examination was much easier after feed had been withheld for twenty-four hours. Subsequent penetration of the rumen has been done repeatedly in the area of adhesions with no evidence of peritoneal infection.

Small wounds open for only a short period of time heal quickly with no aftercare. Stab wounds which have been kept open for several hours are filled with an antibiotic ointment and are allowed to heal with no further treatment, a process that requires approximately seven days.

SUMMARY

A procedure is described to "deperitonealize" a limited area of the rumen so that small openings can be made repeatedly without the danger of peritoneal infection.

From the Department of Veterinary Medicine and Surgery (Ebert); and the Department of Veterinary Physiology and Pharmacology (Roseboom and Dale); School of Veterinary Medicine, University of Missouri, Columbia.

Journal series paper No. 1407 approved by the director of the Missouri Agricultural Experiment Station.

A Comparison of the Effects of Subcutaneous and Intracaudal Vaccination of Sexually Mature Cattle with *Brucella Abortus* Strain 19

DAVID T. BERMAN, D.V.M., Ph.D.; B. A. BEACH, D.V.M.; M. R. IRWIN, Ph.D.

Madison, Wisconsin

DURING the past decade a number of problems concerning the use of *Brucella abortus* strain 19 for the immunization of cattle have assumed considerable importance. Among these problems are the relative merits of vaccination at different ages, the possible advantages of different routes of administration or different amounts of the vaccine suspension, the effects of all or any of these variables upon the resistance engendered, upon diagnostic tests, or reproductive efficiency of vaccinated animals. The experiment described here was designed to furnish information on the effects of vaccination by the intracaudal and subcutaneous routes in sexually mature cattle, including their response to exposure to infection during the third gestation.

MATERIAL AND METHODS

In the spring and summer of 1946, 52 unvaccinated grade Holstein-Friesian heifers ranging in age from approximately 12 to 20 months, and negative to the agglutination test, were purchased from various sources. The animals were held in isolation for four months during which all were found to be negative on monthly agglutination tests. Twelve of the animals were in early pregnancy at the time they were purchased.

Breeding by natural service was begun on Nov. 7, 1946, and animals were bred as they were observed to be in estrus. All but 1 (C) of the cattle were assigned to two groups for vaccination, and efforts were made to have approximately equal numbers of open and pregnant heifers in each group. On Dec. 23, 1946, the animals in group 1 were inoculated subcutaneously with 5 ml. of *Br. abortus* strain 19 which had been freshly prepared in this laboratory according to the standards of the U. S. Bureau of Animal Industry for this vaccine. At the same time, the animals in group 2 received 0.25 ml. of the same vaccine suspension inoculated into the dense fibrous tissue at the tip of the tail as described by Campbell and Rodwell.[1]

Because of space limitations in barn 1, 12 of the vaccinated animals (31, 32, 33, 34, 36, 37, 38, 39, 41, 42, 46, 52) were kept in barn 2 with another herd of animals on a calfhood vaccination experiment which has been described previously.[2, 3] Most of the 12 heifers of the present trial were in the first gestation period at the time when 4 of the cows, with which they were associated in barn 2, became infected in a spontaneous outbreak of brucellosis which has been discussed elsewhere.[2] There was no evidence of infection among the 12 cattle vaccinated as young adults.

All animals were bred for the second pregnancy by natural or artificial insemination and by natural service for the third.

Six unvaccinated yearling heifers were added to the group in barn 1, to serve as controls, at the termination of the first pregnancies of the vaccinated animals. All of these control animals were in the third trimester of their first pregnancies at the time the vaccinated animals in barn 1 were calving for the second time.

All animals were given blood tests at monthly intervals and more frequently immediately after vaccination. End titers of serums were determined by means of the conventional tube agglutination test, using antigen supplied by the Bureau of Animal Industry.

At each parturition, including those after exposure to a virulent culture in 1950, cultures and guinea pig inoculations for the presence of Brucella organisms were made from uterine materials and udder secretions. The materials examined and the methods employed have been described earlier.[3]

During the period between vaccination in 1946 and exposure in 1950, 10 animals were removed from the herd because of infertility, mastitis, or other causes.

On Jan. 16, 1950, the animals described in the present report, as well as those which have been previously described,[3, 4] were exposed with 12×10^6 organisms of *Br. abortus* strain 2308 by the conjunctival route. The details of the preparation of the exposure culture, the reasons for the selection of the numbers of organisms given, and the response of the control and other experimental groups have been presented in detail in the earlier reports.[3, 4]

From the Department of Veterinary Science (paper No. NS 137) and the Department of Genetics (No. 550) Wisconsin Agricultural Experiment Station, University of Wisconsin, in cooperation with the Bureau of Animal Industry, U.S.D.A. Published with the approval of the director of the station.

The authors gratefully acknowledge the advice and assistance given by the following members of the University Brucellosis Research Committee: Professors C. A. Brandly, S. H. McNutt, and E. V. Morse. Technical assistance has been given by Dr. Lois M. Jones, Mrs. June Eberts, and Mr. Archie Simonson. We also express our appreciation for their capable assistance to the herdsmen: O. A. Jacobson, F. Blakely, A. Marotz, and H. Lehnherr.

Of the 41 experimental animals (tables 3 and 4) which were exposed to infection, 2 were not pregnant; the others were between twenty-nine and 170 days in gestation with a mean of 139 days at the time of exposure.

Following exposure, agglutination tests were made at approximately ten-day intervals. At the termination of each pregnancy, cultures and guinea pig inoculations were made. Repeated attempts were made to isolate Brucella organisms from those cows which failed to yield the organism at parturition but had suspicious or positive serum or whey agglutination reactions. Nineteen animals were bred after the postexposure parturition and slaughtered at times when most of them were in advanced pregnancy. Cultures and guinea pig inoculations were made from uterine contents and various lymph nodes of these cattle, which had given serological or other evidence of infection but from which it had not been possible to isolate Brucella organisms at parturition or from repeated milk samples.

EXPERIMENTAL RESULTS

Agglutinin Response to Vaccination.—The distribution of negative, suspect, and reactor animals among the various groups at 3, 6, 9, 12, 18, 25, and 35 months after vaccination as young adults is shown in table 1. Significant differences in the proportions of animals in the various classes between those cattle vaccinated intracaudally with 0.25 ml. of strain 19, and those vaccinated subcutaneously with 5 ml. were not observed. Similarly, differences in the maximum titers attained by the animals vaccinated subcutaneously and intracaudally were not apparent. Although the numbers in the sub-groups are too small for analysis, there is a suggestion that the stage of pregnancy at the time of vaccination was directly related to the persistence of diagnostic agglutinin titers. The majority of animals remained reactors or suspects through two years following vaccination.

Effect of Vaccination on First Pregnancy.—The mean elapsed times from vaccination to conception, or from conception to vaccination, in the various groups as well as the outcomes of the pregnancies are given in table 2. Evidence of interference with conception by vaccination shortly before service, or of an effect of vaccination shortly after service, on the outcome of the pregnancy was not obtained. Three of the 12 animals vaccinated in advanced pregnancy (ca. 5 months) aborted. All 3 had been vaccinated intracaudally. A strain of *Br. abortus* which did not require added CO_2 for primary isolation, and which had the same sensitivity to thionine blue as strain 19,[5] was recovered from 1 of these fetuses. *Brucella abortus* was not isolated from fetuses, placentas, or milk of the other cows which aborted.

One animal vaccinated intracaudally during early pregnancy delivered a dead fetus at term. *Brucella abortus* was not isolated. Eleven of the 49 vaccinated heifers retained the placenta at the first calving.

Of the 46 animals bred for the second pregnancy, 42 conceived and all but 1 delivered vigorous calves at term. Cow 40 aborted after 240 days of gestation, but Brucella organisms could not be recovered from the fetus, placenta, or numerous milk samples.

Of the 42 animals bred for the third pregnancy, all but 1 conceived. On Dec. 25, 1949, one month before exposure to a virulent culture, cow 49 in barn 1 aborted twin fetuses on the 143rd day of pregnancy. This animal had had pyelonephritis in May, 1949, and had made a clinical recovery fol-

TABLE 1—The Agglutination Test Status of Cattle at Various Intervals After Vaccination with Strain 19

	Months after vaccination																							
	3			6			9			12			18			25			35					
Vaccination method	R	S	N	R	S	N	R	S	N	R	S	N	R	S	N	R	S	N	R	S	N			
Subcutaneous (5 ml.)																								
Open	7	3	0	2	7	1	1	9	0	1	7	1	0	8	1	0	7	2	0	2	7			
Early pregnancy	6	5	0	4	7	0	1	10	0	1	9	0	1	7	2	0	7	3	0	3	6			
Advanced pregnancy	2	4	0	3	3	0	2	4	0	2	2	0	2	2	0	2	2	0	2	1	1			
Totals	15	12	0	9	17	1	4	23	0	4	18	1	3	17	3	2	16	5	2	6	14			
Intracaudal (0.25 ml.)																								
Open	3	6	1	1	7	2	0	8	2	0	5	3	0	5	3	0	5	3	0	0	8			
Early pregnancy	5	3	1	2	6	1	1	5	2	1	5	2	2	6	1	0	7	1	0	3	5			
Advanced pregnancy	3	3	0	2	4	0	1	5	0	1	5	0	1	4	0	1	2	2	1	1	2			
Totals	11	12	2	5	17	3	2	18	4	2	15	5	3	15	4	1	14	6	1	4	15			
Herd totals	26	24	2	14	34	4	6	41	4	6	33	6	6	32	7	3	30	11	3	10	29			

R—reactors (titer 1:100 or higher); S—suspects (incomplete or complete agglutination at 1:50 or incomplete at 1:100); N—negative (no agglutination at 1:50).

TABLE 2—The Effects of Vaccination with Brucella Abortus Strain 19 on the First Pregnancy of Cattle Vaccinated as Young Adults

Vaccination method	Days vaccination to service		Days service to vaccination		Number conceived	Abnormal terminations pregnancy	Retained placenta	Strain 19 recovered
	Range	Mean	Range	Mean				
Subcutaneous								
Open	7 to 98	41	.....	....	9/10	0/9	1	0
Early pregnancy	.....	....	3 to 44	28	10/10	0/10	1	0
Advanced pregnancy	.....	....	?	150?	6/6	0/6	2	0
Totals					25/26	0/25	4	0
Intracaudal								
Open	7 to 91	39	.....	....	10/11	0/10	1	0
Early pregnancy	.....	....	3 to 46	26	8/8	1/8	2	0
Advanced pregnancy	.....	....	?	150?	6/6	3/6	4	1
Totals					24/25	4/24	7	1
Herd totals					49/51	4/49	11	1

lowing intensive penicillin therapy, although the ureters remained enlarged. Brucella organisms were not isolated on direct culture of fetal tissues or placenta, nor from colostrum and repeated milk samples. Four guinea pigs, which had been inoculated with a pool of fetal stomach contents and placenta, had negative agglutinin titers when destroyed four weeks after inoculation. One colony of *Br. abortus* appeared on a plate inoculated with a prefemoral lymph node from 1 of these guinea pigs. All other cultures from guinea pig spleens, livers, testes, and lymph nodes were sterile. The strain of *Br. abortus* isolated did not require added CO_2 for growth, was pathogenic for guinea pigs, and was resistant to concentrations of thionine blue inhibitory to strain 19. There was no increase in the agglutinin titer of the cow's serum during the next month before she was exposed to a virulent culture. Forty days after that exposure the agglutinin titer increased from a pre-exposure level of 1:100 to 1:400. It is the authors' opinion that the abortion was not caused by Brucella infection, and that the single Brucella colony isolated from guinea pig tissue was a laboratory contaminant. Prior to exposure none of 6 unvaccinated controls was found to be infected.

TABLE 3—The Response of Cows, Vaccinated Subcutaneously, to Brucella Exposure During the Third Gestation

Cow (No.)	Titer at exposure	Days service to exposure	Days exposure to parturition	Duration of pregnancy (days)	Fate of calf L or D*	Brucella recovery			Maximum** titer post-exposure
						Placenta or fetus	Colostrum	Milk	
23	25	138	77	215	D	+	—	3/3†	12,800
30	—§	Nonpregnant	.....	.....	....	0‡	0	0	200
31	—	105	183	288	L	—	—	0/9	100
33	p 50	147	132	279	L	—	—	0/17	1,600
37	—	29	222	251	L	—	—	0	100
42	p 25	158	127	285	L	—	—	0/15	200
46	50	109	178	287	L	+	—	0/11	1,600
52	—	162	125	287	L	—	—	0	50
60	—	167	121	288	L	—	—	0	50
12	50	157	132	289	L	—	—	0/9	200
14	p 50	159	115	274	L	+	—	0/2	6,400
17	25	151	57	208	D	+	—	0/4	12,800
25	25	89	192	281	L	—	—	0/1	100
50	p 25	166	62	228	D	+	—	1/4	6,400
56	—	155	127	282	L	—	—	0/1	400
58	—	141	139	280	L	—	—	0/3	200
59	p 100	152	128	280	L	—	—	0/13	400
87	—	170	111	281	L	—	—	0	100
19	100	160	118	280	L	—	—	0/10	1,600
35	p 50	141	140	281	L	—	—	0/3	100
44	100	158	116	274	L	—	—	0/11	800
48	25	155	133	288	L	—	—	0/8	200
	Means	141	131	272	Totals	5	0	4/134	

* L—calf living; D—calf dead; **—highest dilution of serum with complete or incomplete agglutination; §—negative at 1:25 dilution; p—partial agglutination at dilution; †—number of times Brucella organisms recovered/number of times quarter samples cultures; ‡—no cultures made.

Cows 23 to 60 vaccinated subcutaneously when nonpregnant; cows 12 to 87 vaccinated subcutaneously during early pregnancy; cows 19 to 48 vaccinated subcutaneously when in advanced pregnancy.

Response to Exposure.—The details of the response of the animals vaccinated subcutaneously are presented in table 3. Four of the animals in this group aborted (17, 23, 37, 50). *Brucella abortus* was recovered from all but cow 37, which attained a maximum agglutinin titer of 1:100. It was not possible to obtain milk samples from this cow because the mammary gland had been sclerosed with acriflavine following traumatic mastitis during the previous lactation. Cultures from tissues obtained at necropsy failed to yield Brucella organisms. It was concluded that the abortion was not associated with brucellosis.

Brucella organisms were recovered from 2 additional animals (14, 46) which had been vaccinated subcutaneously and delivered vigorous calves at term. Each of these animals had high titers of agglutinins.

It may be seen in table 3 that all but 2 of the 22 animals vaccinated subcutaneously developed agglutinin titers of 1:100 or higher at some time during the postexposure period, while infection could be demonstrated in only 5 animals. Only the maximum titer observed is recorded, and in many instances the titers fell below 1:100 during the postexposure period. Most of the animals were suspects, and some remained reactors throughout the observation period.

The details of the response of the animals vaccinated intracaudally are presented in table 4. One animal (43) aborted and Brucella organisms were recovered from the placenta and fetus. This cow had a high titer of agglutinins. Two additional animals in this group (13, 55) which delivered vigorous calves at term were demonstrated to have mammary gland infection. The maximum agglutinin titers attained by these infected cattle were 1:200 and 1:100, respectively.

All but 4 of the 19 animals vaccinated intracaudally developed agglutinin titers of 1:100 or higher during the postexposure period. As in the group vaccinated subcutaneously, the titers receded below the reactor level in many instances, but most of the uninfected animals were suspects and some remained reactors throughout the observation period.

The response of the unvaccinated control animals to exposure have been published in detail elsewhere [3] and these data are summarized in table 5 of the present paper. Included in the table for comparison are data obtained from unvaccinated animals fed a trace-mineral supplement,[4] and from a group of 14 animals vaccinated at 8 months of age and exposed in the third pregnancy. The cattle in each of these five groups were exposed to infection at the same time and in the same manner.

TABLE 4—The Response of Cows, Vaccinated Intracaudally, to Brucella Exposure During the Third Gestation

Cow (No.)	Titer at exposure	Days service to exposure	Days exposure to parturition	Duration of pregnancy (days)	Fate of calf L or D*	Brucella recovery			Maximum** titer post-exposure
						Placenta or fetus	Colostrum	Milk	
11	25	158	127	285	L	—	—	0/12†	100
15	—§	163	125	288	L	—	—	0/3	100
32	p 25	99	188	287	L	—	—	0/6	200
34	25	100	178	278	L	—	—	0/6	1,600
36	25	131	154	285	L	—	—	0	50
38	—	67	223	290	L	—	—	0	50
45	—	140	142	282	L	—	—	0	50
54	25	140	132	272	L	—	—	0/11	200
13	—	155	127	282	L	—	—	2/8	200
16	p 25	148	136	284	L	—	—	0/2	400
20	p 50	149	133	282	L	—	—	0/9	400
24	25	102	176	278	L	—	—	0/5	400
55	p 25	154	130	284	L	—	—	1/1	100
57	—	152	121	273	L	—	—	0/3	50
61	p 50	124	159	283	L	—	—	0/8	400
41	p 25	152	127	279	L	—	—	0/18	100
43	p 25	163	58	221	D	+	—	0/3	6,400
47	p 50	150	136	286	L	—	—	0/6	200
49	100	Nonpregnant when exposed, *see* text.				0‡	0	0	400
	Means	136	143	279	Totals	1	0	3/101	

* L—calf living; D—calf dead; **—highest dilution of serum with complete or incomplete agglutination; §—negative at 1:25 dilution; p—partial agglutination at dilution; †—number of times Brucella organisms recovered/number of times quarter samples cultures; ‡—no cultures made.

Cows 11 to 54 vaccinated intracaudally when nonpregnant; cows 13 to 61 vaccinated intracaudally during early pregnancy; cows 41 to 49 vaccinated intracaudally when in advanced pregnancy.

DISCUSSION

The Agglutinin Response to Vaccination. — Manthei [6] and Gregory [7] have recently reviewed studies on the agglutinin response of cattle of various ages following administration of strain 19 in various amounts by the subcutaneous, intracutaneous, and intracaudal routes. Their studies indicate (1) that the agglutinin titer attained is directly related to the amount of strain 19 administered by any one method; (2) that the agglutinin response to 1 ml. of strain 19 injected intracaudally is significantly greater than to 5 ml. injected subcutaneously; (3) that the response to 0.2 ml. of strain 19 intracaudally is of the same order as to 5 ml. subcutaneously; (4) that the agglutinin titer and the time required for a return to the negative status are directly related to the age of the animal at the time of vaccination for any one method of administration of the vaccine.

In the present investigation, significant differences were not observed in the agglutinin response to 0.25 ml. of strain 19 injected intracaudally or to 5 ml. injected subcutaneously, confirming the observation of Campbell and Rodwell.[1] Differences in the time required to return to a negative status were not apparent between the groups of animals vaccinated by the two methods. Although the number of animals in each of the sub-groups is relatively small, there is a suggestion that the time required to return to a negative status is related to the stage of pregnancy at the time of vaccination. Recession of titers was most rapid in animals which were open at the time of vaccination, less so in those in early pregnancy, and slowest in those in advanced pregnancy. Moore and Mitchell [8] in field investigations of vaccination of sexually mature cattle in herds free of brucellosis found that pregnancy at the time of vaccination was conducive to persistence of the vaccinal reaction.

Response to Induced Infection. — It is clear from the data presented that, in comparison with unvaccinated control animals, both of the vaccinated groups had a considerable degree of resistance to induced infection. This is reflected both in the abortion and infection rates. Significant differences in resistance referable to the mode of administration of the vaccine were not observed. Similar results were obtained by Buddle [9] and Gregory [10] in trials in which groups of calves were vaccinated with 5 ml. subcutaneously or 1 ml. intracaudally and exposed to infection during the first pregnancy, and first and second pregnancies, respectively. McDiarmid [11] has reported that vaccination of heifers near breeding age with 1 ml. intracaudally conferred a resistance to exposure during the first pregnancy comparable with that engendered by vaccination with 5 ml. subcutaneously.

As shown in table 5, significant differences were not apparent in resistance to infection between the two groups vaccinated as young adults in the present trial and the group of animals vaccinated at 8 months of age. All three groups were exposed to infection together during the third gestation period with the same number of organisms of the same strain. It has been rather generally accepted that the resistance engendered by vaccination is greater in animals vaccinated when adolescent or mature than in calves vaccinated when younger than 9 months of age. This impression apparently is based almost entirely upon the limited trial made by Haring and Traum [12] in which 6 of 9 animals vaccinated at 6 to 8 months of age, and 5 of 14 vaccinated as mature animals became infected following

TABLE 5—Summary of the Response of Vaccinated and Unvaccinated Cattle to Exposure with 12 x 10^6 Brucella Abortus Strain 2308

Groups of cattle	Abnormal termination pregnancy		Full term parturition		Nonpregnant		Reactors	Total animals per group
	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected		
Controls	14	1	3	0	2	2	22	22
Trace mineral-fed unvaccinated	12	0	1	3	1	2	17	19
Calfhood vaccinated	3	1	0	10	0	0	8	14
Adult vaccinated subcutaneously	3	1	2	15	0	1	20	22
Adult vaccinated intracaudally	1	0	2	15	0	1	15	19

artificial exposure. These differences are not significant ($\chi^2 = 2.1$, $p = 0.14$). The results of the present trial support the concept that vaccination of calves 8 months of age engenders resistance comparable with that produced by vaccination of sexually mature heifers. Similar results have been reported by Manthei *et al.*[13] There appears to be no evidence that the disadvantage of persistent vaccinal agglutinins which are characteristic in animals vaccinated when mature are compensated for by increased resistance. The diagnostic difficulty presented by persistent vaccinal reactions in animals vaccinated as adults in herds free of brucellosis would be greatly increased in herds in which exposure to infection occurs.[14] Cattle, vaccinated as adults or vaccinated as calves and revaccinated as adults,[3] which can not be demonstrated to be infected following experimental conjunctival exposure, have a greater tendency to develop titers of agglutinins above the diagnostic level than do animals vaccinated as calves. Consideration of these facts would seem to make it mandatory to maintain strict limitations on the use of strain 19 in sexually mature animals in order to prevent confusion in organized control programs.

The present trial, as well as those previously cited, indicates no advantage of the intracaudal method of administration of strain 19 over the conventional technique. Although it is of interest, and may be of significance in some areas in terms of economy of vaccine production, that 0.25 ml. of strain 19 intracaudally was of equal efficacy as 5 ml. subcutaneously, the large dose does constitute a safety factor in insuring that the animals receive at least a minimal number of viable strain 19 cells under field conditions.

SUMMARY

Sexually mature cattle were vaccinated with either 5 ml. of *Brucella abortus* strain 19 subcutaneously or 0.25 ml. intracaudally. Differences attributable to the mode of vaccination, in the titer of agglutinins, or in the rate of disappearance of agglutinins, were not apparent. There was a greater tendency for animals to retain significant titers of agglutinins if they had been pregnant at the time of vaccination regardless of the method employed.

The animals were exposed during the third pregnancy by the conjunctival route with 12×10^6 organisms of *Br. abortus* strain 2308. Both groups of vaccinated animals were significantly more resistant than unvaccinated control cattle, but differences between the groups were not apparent.

References

[1] Campbell, A. D., and Rodwell, A. W.: The Relationship of Dosage and Site of Inoculation to the Agglutinin Response to *Brucella Abortus* Strain 19 Vaccine. A Comparison of the Subcutaneous, Intracutaneous, and Intracaudal Routes. J. Comp. Path. and Therap., *55*, (1945): 277-289.

[2] Berman, D. T., and Beach, B. A.: Studies on Repeated Vaccination of Cattle with *Brucella Abortus* Strain 19. I. The Agglutinin Response of Animals Vaccinated as Calves and Revaccinated as Young Adults. Am. J. Vet. Res., *10*, (1949): 208-213.

[3] Berman, D. T., Beach, B. A., and Irwin, M. R.: Studies on Repeated Vaccination of Cattle with *Brucella Abortus* Strain 19. III. The Response of Vaccinated and Revaccinated Cattle to Conjunctival Exposure with a Virulent Strain of *Brucella Abortus* During the Third Gestation Period. Am. J. Vet. Res., *12*, (1952): 351-358.

[4] Berman, D. T., Phillips, P. H., and Brandly, C. A.: The Failure of a Trace Mineral-Supplemented Ration to Influence the Course of Bovine Brucellosis. J.A.V.M.A., *121*, (1952): 46-50.

[5] Levine, H. B., and Wilson, J. B.: The Identification of *Brucella Abortus* Strain 19 by Dye Bacteriostasis. J. Infect. Dis., *84*, (1949): 10-14.

[6] Manthei, C. A.: Evaluation of Vaccinal Methods and Doses of *Brucella Abortus* Strain 19. Proc. U. S. Livestock San. A., (1952): 115-125.

[7] Gregory, T. S.: A Comparison of the Effects of Intracaudal and Subcutaneous Vaccination of Calves with *Brucella Abortus* Strain 19. 4. A General Assessment of Results Three Years After Vaccination. Austral. Vet. J., *29*, (1953): 117-121.

[8] Moore, T., and Mitchell, C. A.: Vaccination of Sexually Mature Cows with *Brucella Abortus* Strain 19 Vaccine. I. Negative Cows Contained in Negative Dairy Herds. Canad. J. Comp. Med., *12*, (1948): 278-283.

[9] Buddle, M. B.: Immunity in Cattle Vaccinated with *Brucella Abortus* Strain 19 by the Subcutaneous and Intracaudal Routes During Calfhood. Austral. Vet. J., *24*, (1948): 262-271.

[10] Gregory, T. S.: A Comparison of the Effects of Intracaudal and Subcutaneous Vaccination of Calves with *Brucella Abortus* Strain 19. 2. Immunity During the First Pregnancy in the Second Year After Vaccination. Austral. Vet. J., *28*, (1952): 194-200.

[11] McDiarmid, A.: A Comparison of the Immunity Produced in Cattle by the Inoculation of *Brucella Abortus* Strain 19 Intradermally, Intracaudally, and Subcutaneously. Vet. Rec., *62*, (1950): 361-364.

[12] Haring, C. M., and Traum, J.: The Effect of *Brucella Abortus* Strain 19 on Cattle of Various Ages and its Bearing on Adult Cattle Vaccination. Proc. U. S. Livestock San. A., (1943): 42-46.

[13] Manthei, C. A., Mingle, C. K., and Carter, R.W.: Duration of Immunity to Brucellosis Induced in Cattle with Strain 19 Vaccine. Proceedings Book, A.V.M.A., (1951): 128-142.

[14] Moore, T., and Mitchell, C. A.: Vaccination of Sexually Mature Cows with *Brucella Abortus* Strain 19 Vaccine. II. Negative Cows in Infected Dairy Herds. Canad. J. Comp. Med., *14*, (1950): 209-214.

Bovine Blood Serum Concentrations of Terramycin (Oxytetracycline) Following Intravenous and Intramuscular Administration

H. W. YODER, Jr., B.S., and R. A. PACKER, D.V.M., Ph.D.

Ames, Iowa

ALTHOUGH terramycin® (oxytetracycline) has been used in combating animal infections of various kinds since its discovery in 1950, little is known regarding the levels of the drug maintained in the blood serum during therapy. Hobby *et al.*[1] devised assay procedures and reported on serum levels of terramycin in rabbits and dogs, and urine levels in dogs. Schipper and Petersen[2] recently reported on bovine blood serum, milk, and urine levels of terramycin in cattle following intravenous, intramuscular, and intramammary administration.

The work reported in this paper concerns the use of a turbidimetric assay technique in determining the levels of terramycin in bovine blood serum at various time intervals following its administration intravenously and intramuscularly at different dosages.

PROCEDURE

Kersey[3] reported on a turbidimetric assay technique for terramycin which employed a Lumetron Colorimeter (model 402-E) and *Klebsiella pneumoniae* as the test organism. In this work, a turbidimetric assay technique similar to that described by Kersey was used. A Coleman No. 7 photoelectric nephelometer was employed to determine the turbidity of the bacterial suspensions. This assay is based on the fact that a given amount of terramycin will limit the growth of a specified bacterial suspension, under standard conditions, in proportion to the amount of terramycin present.

Staphylococcus aureus, strain 209, was used as the assay organism. Single colonies selected from blood agar plates were inoculated into tubes of nutrient broth containing four glass beads and were incubated at 37 C. for about twenty-four hours. The next tube of broth was inoculated with 1 loopful from the previous tube and so on daily. The test organism was plated on blood agar and reisolated from single colonies once each week. Care was taken so that no culture was used after it had contacted any terramycin. Each daily culture was of moderate turbidity and with vigorous shaking the glass beads helped to provide a uniform suspension of the organisms in the broth.

Enough culture suspension was added to 6 to 8 ml. of nutrient broth in a nephelometer tube (previously set at zero in the nephelometer) to give a turbidity reading of 7.0 plus or minus 0.1 units on the galvanometer scale of the nephelometer. Then the tube of prepared inoculum was placed in a beaker of ice water to retard further growth until the time of inoculation of the assay tubes. The tube of inoculum should not be prepared until all of the assay tubes have been set up with all of the constituents and are ready to be inoculated.

TABLE 1—Constituents of Nephelometer Tubes Prepared for Turbimetric Assay of Terramycin® (Oxytetracycline) Levels in Bovine Blood Serum

Tube No.	Standard terramycin serum, 1 μg./ml. (ml.)	Normal serum (ml.)	Brain-heart infusion broth* (ml.)	Inoculum (ml.)	Terramycin μg./ml. of tube contents (ml.)
1	1.00	0.00	6.70	0.30	0.1250
2	0.80	0.20	6.70	0.30	0.1000
3	0.60	0.40	6.70	0.30	0.0750
4	0.40	0.60	6.70	0.30	0.0500
5	0.30	0.70	6.70	0.30	0.0375
6	0.20	0.80	6.70	0.30	0.0250
7	0.10	0.90	6.70	0.30	0.0125
8	0.00	1.00	6.70	0.30	Control

* Difco dehydrated.

The various nephelometer tubes of the standard terramycin series were set up according to table 1.

The sterile, cotton-plugged nephelometer tubes were arranged in racks and the constituents were pipetted into them. After pipetting the standard terramycin serum,* enough normal serum was added to each tube to make a total of 1.0 ml. of serum per tube. Then 6.70 ml. of brain-heart infusion broth was added to each tube. The 0.30 ml. portion of inoculum, which is added later, makes a total of 8.0 ml. of constituents per nephelometer tube. Each tube in the standard terramycin series then contains a known amount of terramycin per milliliter of tube contents. Tube 8 is the control tube which contains no terramycin.

The serum samples to be assayed were set up in

Mr. Yoder is a senior student in the Division of Veterinary Medicine at Iowa State College, Ames. Dr. Packer is professor and head of the Department of Veterinary Hygiene, Division of Veterinary Medicine, Iowa State College, Ames.

This work was supported in part by a grant from the Charles Pfizer Co., Brooklyn, New York.

* The standard terramycin is prepared to contain 1.0 μg. of terramycin per milliliter of serum. It is prepared by various dilutions with sterile normal bovine serum, using a 20,000 μg. vial of assay quality terramycin hydrochloride obtained from the Pfizer Antibiotic Diagnostic Kit supplied by Charles Pfizer and Co., Inc.

TABLE 2—Summary of Average Figures for Terramycin® (Oxytetracycline) Serum Levels

	Micrograms of terramycin per milliliters of serum													
Hours after injection	½	1	2	4	12	24	½	1	2	4	12	24	36	48	72
Intravenous							(36)								
1 Gm., 1 dose, ave. of 5 trials	4.99	3.76	2.66	1.89	0.818	0.245	0.133	.....	.....	.....	.....	.....	.....	.....	.....
1 Gm., 2 doses, ave. of 2 trials	4.88	3.72	2.58	1.81	0.540	0.209	Inject.	5.20	3.85	2.68	1.89	0.718	0.294	0.120	0.099	0.083
2 Gm., 2 doses, ave. of 2 trials	10.64	7.55	5.55	3.56	1.45	0.381	Inject.	11.04	7.12	5.32	3.94	1.44	0.394	0.178	0.139	0.117
Intramuscular							(36)								
1 Gm., 1 dose, ave. of 5 trials	.....	1.71	1.88	1.80	1.25	0.600	0.240	.....	.....	.....	.....	.....	.....	.....	.....
							(36)	(48)	(60)	(72)					
1 Gm., 1 dose, 1 trial	.....	1.71	1.77	1.44	0.930	0.400	0.180	0.140	0.130	0.100	.....	.....	.....	.....	.....
1 Gm., 2 doses, ave. of 2 trials	.....	1.63	1.90	1.80	1.27	0.640	Inject.	.....	1.83	2.33	2.16	1.34	0.690	0.300	.....
							(36)								
2 Gm., 1 dose, ave. of 5 trials	.....	2.72	2.92	2.85	2.04	0.930	0.430	.....	.....	.....	.....	.....	.....	.....	.....
2 Gm., 2 doses, ave. of 2 trials	.....	2.10	2.24	2.20	1.56	0.870	Inject.	.....	2.30	2.77	2.99	2.33	1.19	0.630	0.320	0.160

a manner similar to that of the standard terramycin tubes. The amount of sample serum to be used had to be determined for each tube because the assay technique employed made it necessary that any given tube contain from about 0.125 to 0.025 μg. of terramycin per milliliter of total volume. Thus, it is apparent that several dilutions had to be used in order that a given serum sample could be accurately assayed. Some serum samples contained so much terramycin that it was necessary to use only 0.20 ml. of a 1: 7 dilution and then 0.80 ml. of normal serum was added. After a few samples had been assayed, it became rather easy to predict the amounts of terramycin in a given serum sample, and fewer tube dilutions were needed in order to assay such samples.

The 6.70-ml. portions of brain-heart infusion broth were added to each of the sample assay tubes and then all of the nephelometer tubes were placed in a water bath at 37 C. Then the inoculum was prepared as previously described. After the eight tubes in the standard terramycin series were withdrawn from the water bath, they were inoculated with 0.30 ml. of inoculum, and read in the nephelometer before being replaced in the water bath to be timed. This procedure was repeated for each set of eight or nine nephelometer tubes of the sample series. All tubes were incubated exactly four hours, then removed in the order placed in the water bath, shaken, and read in the nephelometer.

The amount of terramycin per milliliter of original serum was determined by comparing the turbidity of the sample tubes with the turbidity of the standard terramycin series tubes. The increase in galvanometer units following the incubation period represents the increase in turbidity for each tube. A standard curve was prepared by plotting the turbidity of the standard series tubes along the abscissa and the known amounts of terramycin per milliliter of the total contents in those tubes along the ordinate. By plotting the galvanometer readings of the sample tubes along the prepared curve, the concentration of terramycin per milliliter of total tube contents was determined by reading down to the ordinate. That figure multiplied by the dilution factor for each sample tube gives the micrograms of terramycin per milliliter of the original serum sample.

The terramycin levels of the various samples could be re-assayed on different days with a fairly high degree of accuracy. Although this assay technique was time consuming, the accuracy and reproducibility of the results justified its use.

Procedure for Administration of Terramycin.—The practicing veterinarian was kept in mind while this project was designed and conducted. No particular attempt was made to use doses according to exact body weight. Instead, several grade dairy cattle were used which weighed approximately 1,000 lb. each. One- and 2-Gm. doses of terramycin were administered intravenously or intramuscularly. Where more than one dose was given, a 24-

hour interval elapsed between doses. Only one assay series was conducted on a lactating cow; the others were run on cows in late pregnancy.

Terramycin intravenous was diluted with water (U.S.P.) and injected into the jugular vein with a 50-cc. glass syringe at a concentration of 20 mg./ml. over a period of three to four minutes. Terramycin intramuscular was diluted to a concentration of 50 mg./ml. and was injected into the heavy muscles of the neck at 10 to 13 ml. per site.

Blood samples were taken at various time intervals after a given dose of terramycin had been administered; also a control sample was taken before each injection. Each blood sample was aseptically withdrawn from the jugular vein, using a 5- or 10-cc. glass syringe, and deposited into a sterile tube. The tubes of blood were refrigerated for no longer than one day before the serum was withdrawn, transferred into other tubes, rubber stoppered, and frozen until assayed.

The frozen serum containing terramycin was found to maintain its potency for at least several weeks. Several samples which had been frozen for two, three, and four weeks were found to be fully potent. One tube of standard assay terramycin diluted to 10 μg./ml. of serum was compared with a new standard nine weeks later and had lost none of its original potency, even though it had been thawed and refrozen on several occasions. However, the serum samples were routinely assayed within several days after collection.

The normal bovine serum was Seitz filtered before being used. The serum samples containing terramycin were not filtered because Hobby *et al.*[4] imply that filtration might retain some terramycin at such low levels when only 2 to 3 ml. samples of serum were used.

RESULTS

The average terramycin concentrations for the two different routes of injection, at the various time intervals after administration, are presented in table 2 and graphically in figures 1 and 2.

It will be noted from figures 1 and 2 that the terramycin concentration in the blood serum is highest in the first thirty minutes following intravenous administration. These high levels decline rapidly, falling to approximately one third of the thirty-minute level by the end of four hours. After

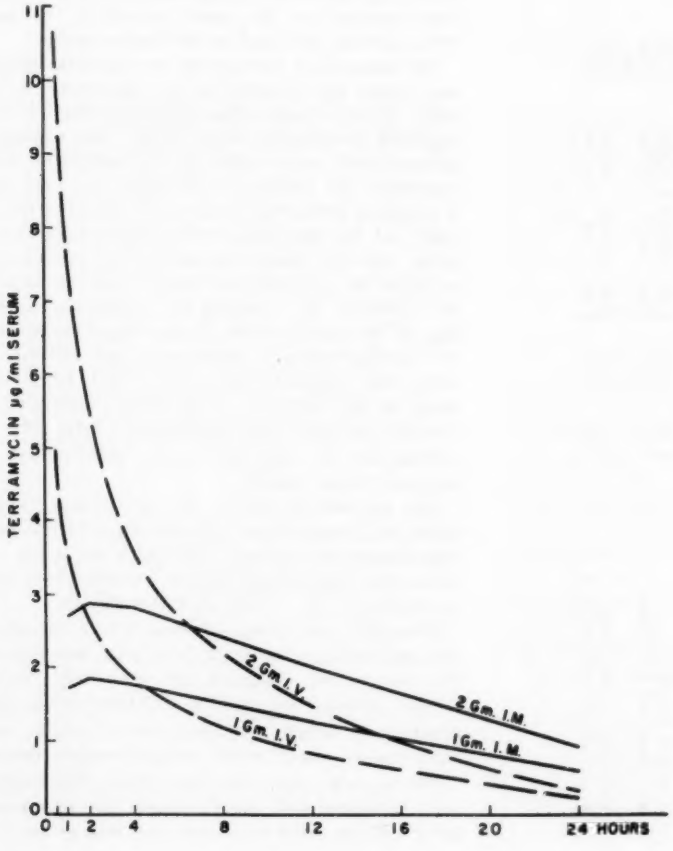


Fig. 1 — Comparison of terramycin® (oxytetracycline) levels in bovine blood serum following the administration of 1-Gm. and 2-Gm. doses intravenously and intramuscularly.

four hours, the concentration declines more gradually with a detectable level remaining for at least seventy-two hours. If a second intravenous dose of terramycin is given twenty-four hours after the first, slightly higher values are obtained showing some additive effect; however, the shape of the curve is not altered.

The peak level of terramycin in serum is not reached until two hours following intramuscular injection. It should be noted that the level following a 1-Gm. intramuscular dose was 1.88 μg. per milliliter, compared to 4.99 μg. per milliliter for the same dose given intravenously. Although the peak levels following intramuscular administration were not as high as those after intravenous injection, the decline was much more gradual and after four to six hours the levels were approximately the same. After eight hours, the intramuscular levels are consistently higher than those which follow intravenous administration. Further, after sixteen hours the levels obtained from a 1-Gm. intramuscular dose are higher than those following a 2-Gm. intravenous dose. The additive effect observed after two intravenous doses spaced twenty-four hours apart was more pronounced with intramuscular administration. At thirty-six hours after terramycin injection, the serum concentrations are low and decline very gradually until seventy-two hours.

Discussion

The exact concentration of terramycin which must be maintained in the blood serum for therapeutic effect can not be stated. Obviously, it would vary depending upon the sensitivity of the invading organism. However, Krantz and Carr [5] have indicated that a range of 0.5 to 1.0 μg. per milliliter in serum is adequate for most therapeutic purposes in adult human beings. Assuming that a similar serum terramycin level in animals is adequate, then on the basis of this report, a dosage schedule can be suggested which would maintain such levels in cattle. It would appear that 1 Gm. of terramycin given intravenously at twelve-hour intervals or 2 Gm. every twenty hours as well as 1 Gm. intramuscularly at 24-hour intervals or 2 Gm. every thirty-six hours would maintain a level of 0.5 μg. or more per milliliter of serum in a 1,000-lb. cow. It is of interest that in this work as little as 0.0125 μg. per milliliter of serum was definitely bacteriostatic to *Staphylococcus aureus*, strain 209. The concentration after seventy-two hours in both the intravenous and intramuscular series was above 0.083 μg. of terramycin per milliliter. Therefore, it may be possible that some benefit may be derived for as long as seventy-two hours after terramycin administration if the invading organism is very sensitive.

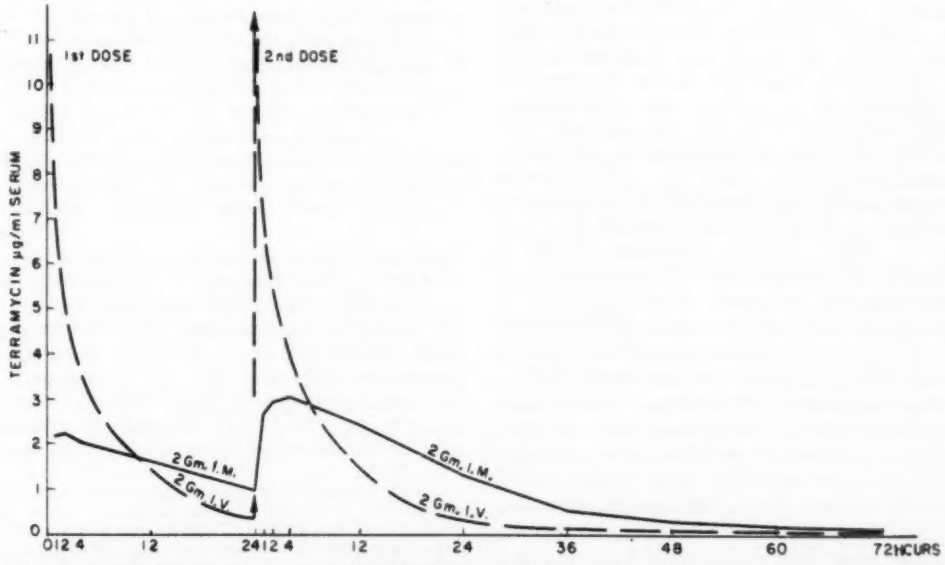


Fig. 2—Comparison of terramycin® (oxytetracycline) levels in bovine blood serum following two 2-Gm. doses administered intravenously and intramuscularly.

SUMMARY

1) A turbidimetric assay technique for bovine blood serum levels of terramycin® (oxytetracycline) is described. This technique affords accurate and reproducible results.

2) The serum levels of terramycin following intravenous administration rise more rapidly, reach a higher peak, and decline more rapidly than levels following the same dose given intramuscularly.

3) The peak terramycin concentrations in serum are higher following a second intramuscular dose administered twenty-four hours after the first dose. This additive effect is less pronounced in the case of intravenous injections.

4) Serum levels following a 2-Gm. intravenous dose are up to twice as high as those obtained with 1 Gm. Two Gm. given intramuscularly give substantially higher serum terramycin levels than a 1-Gm. dose but are not doubled.

5) The parenteral administration of at least 1 Gm. of terramycin into a 1,000-lb. cow will give detectable blood levels for at least seventy-two hours.

References

[1] Hobby, G. L., Reed, W., Rinne, D., Powers, M., D'Ambrosia, Anne: Absorption and Excretion of Terramycin in Animals. Proc. Soc. Exptl. Biol. and Med., *73*, (March, 1950): 511-515.

[2] Schipper, I. A., and Petersen, W. E.: Milk, Blood, and Urine Concentrations of Terramycin Following Intravenous, Intramuscular, and Intramammary Administration. Vet. Med., *48*, (Aug., 1953): 315-318.

[3] Kersey, R. C.: A Turbidimetric Assay for Terramycin. J. Am. Pharm. A., *39*, (May, 1950): 252-253.

[4] Hobby, G. L., Dougherty, N., Lenert, T. F., Hudders, E., and Kiseluk, M.: Antimicrobial Action of Terramycin *in Vitro* and *in Vivo*. Proc. Soc. Exptl. Biol. and Med., *73*, (March, 1950): 503-511.

[5] Krantz, J. C., Jr., and Carr, C. J.: The Pharmacologic Principles of Medical Practice. 2nd ed. The Williams and Wilkins Co., Baltimore, 1951.

New Studies on Comparative Brucella Immunity with Agglutinogenic and Nonagglutinogenic Vaccines

FRANCISCO SIEIRO, D.V.M.

Buenos Aires, Argentina

RESEARCH on bovine brucellosis, on the basis of field use of vaccines, started in Argentina when Rosenbusch,[1] in 1922, reported on the preparation and use of a vaccine to prevent infectious abortion in cattle. The *Brucella abortus* strain employed later proved to be nonagglutinogenic. The use of that vaccine has continued for twenty-one years with more than 1 million cattle vaccinated.[2]

An attempt to evaluate, by artificial exposure, the immunizing potency of this agent in cattle was reported recently by Sieiro and Rosenbusch.[3] The complexity of Brucella immunity became promptly evident, so additional experiments were planned, the results of which are expected to be published in due time. In the present paper, a comparative study is made of the immunity attained in heifers vaccinated with strain 19 (BAI) and the nonagglutinogenic strain "B" (Rosenbusch).

MATERIALS AND METHODS

This work was started in 1951 with 83 heifers of the Shorthorn and the Argentine Holstein-Friesian breeds, with ages ranging from 12 to 14 months. They were kept on premises that had not contained cattle for several years and were thus supposedly free from Brucella infection. At the beginning of the study, all but 1 of the heifers were negative to Wright's serum-agglutination test. The exception was heifer 52, which was positive at first, but became negative prior to artificial exposure.

The 83 animals of the experiment were divided into three groups and placed in separate paddocks with similar conditions as to feed, water, and shelter.

Group A contained 18 heifers which were inoculated subcutaneously on March 27, 1951, with 5 cc. of a vaccine prepared with strain 19 (BAI), containing 10 billion live organisms per milliliter. The vaccine strain proved to be in the smooth phase when analyzed by the usual biological procedures.

Group B consisted of 45 animals vaccinated subcutaneously on April 30, 1951, with 5 cc. of a vaccine prepared from a saline suspension of the nonagglutinogenic *Brucella abortus* strain B, containing 10 billion live organisms per milliliter. Heifer 52 which had previously given a positive agglutination titer was included in this group.

Group C contained 20 heifers, which on March 27, 1951, were tested for agglutinin antibodies with completely negative results, and were used as unvaccinated controls.

On Sept. 1, 1951, 3-year-old bulls, with negative blood tests, were added to each group of heifers. The bulls were renewed (or changed) every two weeks or so, in order to obtain the largest proportion of pregnancies.

The artificial exposure was performed on April 18, 1952. Nonpregnant animals were removed from the experiment, thus reducing group A to 15 animals, group B to 42, and group C to 19.

Artificial exposure was made with a *Brucella abortus* strain of high virulence, the activity of which had been previously verified in heifers and guinea pigs. In order to apply two different infective doses, each of the three groups of heifers was divided into two subgroups as follows: 6 heifers in subgroup A-1, 9 in A-2, 21 in B-1, 21 in B-2, 11 in C-1, and 8 in C-2. Subgroups A-1, B-1, and C-1 received a smaller dose designated "infection I"; subgroups A-2, B-2, and C-2 received a larger dose called "infection II."

The virulent strain was cultured in potato-agar slants at 37.5 C. for forty-eight hours. The culture was then suspended in a peptonized, physiological saline solution, prepared by the technique of De Mello, Danielson, and Kiser,[4] in order to enhance the viability of the organisms. All animals were exposed by placing 0.05 ml. of the infective material under the lower lid of each eye, after which the eyelid was energetically massaged for five to ten seconds. Colony counts of the virulent suspensions performed later in trip'ose-agar plates showed that the lower infective dose (infection I) had been of 5 million organisms (5×10^{-6}) while the higher dose (infection II) had been of 50 million (5×10^{-7}). Both infective doses were stronger than had been originally intended; still, the comparative results are illustrative enough.

The heifers were watched closely after exposure. Each female, with signs of oncoming parturition or abortion, was placed alone in a lot especially provided for births, in order to prevent overinfection in the original lots. Facilities were provided for the collection and delivery of materials to the laboratory for analysis after parturition or abortion. The cows and their calves were later taken to a convalescent lot.

From the Instituto Rosenbusch S. A., Buenos Aires.

The author expresses his appreciation to Drs. Francisco Rosenbusch and Carlos T. Rosenbusch for the facilities provided for the realization of this work, and to Dr. Benjamin D. Blood for his assistance in revising the English manuscript.

TABLE 1—Reactions of Vaccinated and Unvaccinated Pregnant Animals to Exposure to Infection I (5 Million Virulent Organisms)—Groups A-1, B-1, and C-1

Group A[1] — Animals Vaccinated with Strain 19 (BAI)
Pregnancy Time at Exposure 4½-6½ months.

Nº	1951 ♀ 2	3	4	5	6	7	8	9	10	11	12	1952 1	2	3	4	5/5	5/26	PREGNANCY TIME AT EXPOSURE	DATE NB - AB	POST PARTUM AGGLUT.	BACTERIOL. INVEST. FETUS	PLACEN.	COLOST.	IMMUNOLOGIC CLASSIFICATION	OBSERVATIONS
014		V													I			5m.	5/20 NB				NEG	IMMUNE	
054		V													I			4m. 24d.	5/24 NB				NEG	IMMUNE	
063		V													I			4m. 17d.	6/1 NB				NEG	IMMUNE	
839		V													I			6m. 7d.	5/26 WC		POSIT	POSIT		INFECTED	CALF DIED 6/27
983		V													I			5m. 25d.	6/23 AB		POSIT	NEG		INFECTED	
988		V													I			6m.	5/28 AB		POSIT			INFECTED	

Group B[1] — Animals Vaccinated with Strain B (Non Agglutinogenic)
Pregnancy Time at Exposure 4½ - 7½ months.

Nº	1951 ♀ 2	3	4	5	6	7	8	9	10	11	12	1952 1	2	3	4	5/5	5/26	PREGNANCY TIME AT EXPOSURE	DATE NB - AB	POST PARTUM AGGLUT.	BACTERIOL. INVEST. FETUS	PLACEN.	COLOST.	IMMUNOLOGIC CLASSIFICATION	OBSERVATIONS
043			V												I			7m.	6/13 NB			POSIT	POSIT	AB. RESIST.	
50			V												I			7m.	6/12 NB				POSIT	AB. RESIST.	
057			V												I			6m.	7/13 NB			POSIT		AB. RESIST.	
60			V												I			7m. 15d.	6/9 NB				POSIT	AB. RESIST.	
066			V												I			6m.	7/11 NB				NEG	IMMUNE	
69			V												I			7m.	6/13 NB				POSIT	AB. RESIST.	
069			V												I			7m. 15d.	6/3 NB			POSIT	POSIT	AB. RESIST.	
87			V												I			6m. 15d.	7/8 NB				NEG	IMMUNE	
91			V												I			7m. 15d.	6/2 NB			NEG	NEG	IMMUNE	
134			V												I			5m. 15d.	7/20 NB				NEG	IMMUNE	
086			V												I			7m. 15d.	6/7 WC		POSIT		POSIT	INFECTED	CALF DIED 6/9
096			V												I			7m.	6/9 WC		POSIT			INFECTED	CALF DIED 6/9
2			V												I			4m. 15d.	7/8 AB		POSIT			INFECTED	
004			V												I			7m.	6/9 AB		POSIT	POSIT		INFECTED	
05			V												I			7m. 15d.	5/23 AB		POSIT	POSIT		INFECTED	
34			V												I			7m.	6/9 AB		POSIT	POSIT		INFECTED	
94			V												I			7m.	6/6 AB		POSIT			INFECTED	
137			V												I			7m.	6/10 AB		POSIT			INFECTED	
159			V												I			6m. 15d.	6/2 AB		POSIT	POSIT		INFECTED	
819			V												I			7m. 15d.	5/8 AB		NEG		POSIT	INFECTED	
958			V												I			6m.	6/14 AB		POSIT	POSIT		INFECTED	

Group C[1] — Unvaccinated Controls.
Pregnancy Time at Exposure 4 - 6½ months.

Nº	1951 ♀ 2	3	4	5	6	7	8	9	10	11	12	1952 1	2	3	4	5/5	5/26	PREGNANCY TIME AT EXPOSURE	DATE NB - AB	POST PARTUM AGGLUT.	BACTERIOL. INVEST. FETUS	PLACEN.	COLOST.	IMMUNOLOGIC CLASSIFICATION	OBSERVATIONS
024															I			4m.	5/19 NB				NEG	NAT. IMMUNE	
051															I			4m. 25d.	5/23 NB				NEG	NAT. IMMUNE	
020															I			4m. 23d.	5/25 AB		POSIT	POSIT		INFECTED	
023															I			6m 12d.	6/9 AB		POSIT	POSIT		INFECTED	
059															I			5m. 15d.	7/4 AB		POSIT			INFECTED	
623															I			6m.	7/2 AB		POSIT	POSIT		INFECTED	
628															I			6m. 7d.	6/11 AB		POSIT	POSIT		INFECTED	
951															I			5m. 11 d.	7/7 AB		POSIT	POSIT		INFECTED	
955															I			6m. 4 d.	6/14 AB		POSIT			INFECTED	
965															I			4m. 25d.	6/24 AB		POSIT	POSIT		INFECTED	
967															I			6m. 6 d.	6/12 AB		POSIT			INFECTED	

▩ = POSIT. AGGLUT. TITRE 1/100 OR HIGHER
▨ = POSIT. AGGLUT. TITRE 1/50
⁘ = POSIT. AGGLUT. TITRE 1/25
□ = NEGATIVE AGGLUTINATION

V = vaccinated; ♀ = breeding time; I = 5×10^{-6} conjunctival exposure; NB = normal birth; AB = abortion; WC = weak calf.

Blood samples for serum-agglutination tests were taken eighteen and thirty-eight days after exposure, and again approximately thirty days after parturition or abortion of each cow. As considerable importance was assigned to the postpartum test in the final analysis of the experiment, it was repeated three times at monthly intervals.

In cases of normal births the placentas, when available, were submitted for culture tests. Guinea pigs were inoculated with colostrum, subcutaneously and intraperitoneally; male guinea pigs weighing close to 400 Gm., were selected for this purpose. In cases of abortion, a detailed study was made of the lesions in the fetus and cultures were made from the stomach content (abomasum), lungs, spleen, liver, ganglia, and brain, as well as from the placental cotyledons when possible. When these cultures proved negative, guinea pigs were inoculated with colostrum from the aborting cow in the same way as that described for normal births.

Cows which gave birth to weak calves were handled as in normal births; if the calf later died, the usual postmortem examination and culture tests of the various organs were made.

Guinea pigs inoculated with colostrum were killed six to eight weeks later and cultures were made from the spleen, liver, testicle, and ganglia.

The final criterion of infection was established by the isolation of the infective organism in cultures from the placenta, from the fetal or guinea pig organs, or by the rising of postpartum agglutinins to titers of 1: 100 or higher.

EXPERIMENTAL RESULTS

Vaccination with strain 19 (BAI) induced agglutination titers which can be considered normal for this strain, when applied in an environment free of infection (tables 1 and 2).

Strain B of *Brucella abortus* does not produce agglutinins so the status of the animals of groups B-1 and B-2 did not change as far as agglutinin antibodies are concerned. Heifer 52, which had a positive titer of 1 : 50 at the beginning of the experiment, gradually lost the titer and became negative nine months after vaccination.

The unvaccinated controls which were also subjected to monthly agglutination tests proved to be constantly negative up to artificial exposure.

The blood tests performed eighteen and thirty-eight days after exposure showed no significant differences between vaccinated and control groups. It can be stated, however, that agglutination titers induced by artificial exposure were proportional to the intensity of the infective dose administered.

The birth rate in the various groups of animals was influenced by virulent exposure as follows:

Infection I (Table 1).—The animals of subgroup A-1 which received strain 19 vaccine were exposed when pregnant four months and seventeen days to six months and seven days (mean pregnancy stage 162 days). Three animals (50.0 %) calved normally without recovery of the organism, 1 cow giving a postpartum agglutination titer of 1 : 50. One animal (16.6 %) gave birth to a weak calf which died in a few hours, with recovery of the infecting organism from cultures of the placenta and organs of the calf. Two animals (33.3 %) aborted, with recovery of the organism from the fetuses. In these last 3 cows the postpartum agglutination titers were higher than 1 :100.

In subgroup B-1 (strain B vaccine), the pregnancy state, at the time of exposure, varied from four and a half to seven and a half months (mean pregnancy stage 202 days). There were ten normal births (47.6 %), in four of which it was neither possible to recover the infective organism nor to detect an infectious state on the basis of the postpartum agglutination tests. In the other six, Brucella infection was demonstrated both by isolation of the virulent organism and by the rising of agglutination titers. Two animals (9.5 %) gave birth to weak calves which died a few hours later, and 9 animals (42.8 %) aborted, all proving infected in the corresponding bacteriological investigation.

The animals of subgroup C-1 (unvaccinated controls) showed at exposure time pregnancies ranging from four to six and a half months (mean pregnancy stage 165 days). Two animals out of 11 (18.1 %) had normal births, proving negative in the bacteriological research and showing low agglutination titers (1 : 25). Nine cows (81.8 %) aborted with recovery of the organism and a generalized rise in their agglutination titers.

Infection II (Table 2).—The subgroup A-2 animals which received strain 19 vaccine were exposed while pregnant five to six months (mean pregnancy stage 172 days). One animal (11.1 %) calved normally and the virulent organism could not be recovered, while the postpartum agglutination was of a low titer (1 : 25), indicative of an arrested or regressive infectious state. One other animal (11.1 %) also calved normally

TABLE 2—Reactions of Vaccinated and Unvaccinated Pregnant Animals to Exposure to Infection II (50 Million Virulent Organisms)—Groups A-2, B-2, and C-2

Group A^2 – Animals Vaccinated with Strain 19 (BAI)
Pregnancy Time at Exposure 5 - 6½ months.

Nº	1951 2	3	4	5	6	7	8	9	10	11	12	1952 1	2	3	4	5/5	5/28	PREGNANCY TIME AT EXPOSURE	DATE NB - AB	POST PARTUM AGGLUT	BACTERIOL. INVEST. FETUS	PLACEN	COLOST.	IMMUNOLOGIC CLASSIFICATION	OBSERVATIONS
03		V													II			6m.	7/14 NB			NEG	NEG	AB. RESIST	
061		V													II			5m.	6/14 NB				NEG	IMMUNE	
028		V													II			5m. 8d.	7/10 WC		NEG		NEG	INFECTED	CALF DIED 7/12
01		V													II			5m. 10d.	7/22 AB		POSIT			INFECTED	
04		V													II			5m. 26d.	7/5 AB		POSIT			INFECTED	
012		V													II			6m. 15d.	7/14 AB		POSIT			INFECTED	
015		V													II			5m. 25d.	6/22 AB		POSIT	POSIT		INFECTED	
045		V													II			6m.	6/18 AB		POSIT			INFECTED	
940		V													II			5m.	6/5 AB		POSIT	POSIT		INFECTED	

Group B^2 – Animals Vaccinated with Strain B (Non Agglutinogenic)
Pregnancy Time at Exposure 5 - 7½ months.

Nº	1951 2	3	4	5	6	7	8	9	10	11	12	1952 1	2	3	4	5/5	5/28	PREGNANCY TIME AT EXPOSURE	DATE NB - AB	POST PARTUM AGGLUT	BACTERIOL. INVEST. FETUS	PLACEN	COLOST.	IMMUNOLOGIC CLASSIFICATION	OBSERVATIONS
040			V												II			7m.	6/6 NB			POSIT	POSIT	AB. RESIST.	
52			V												II			7m.	6/18 NB			NEG	NEG	IMMUNE	
618			V												II			7m. 15d.	5/30 NB				POSIT	AB. RESIST.	
013			V												II			7m. 15d.	5/20 WC			POSIT		INFECTED	CALF DIED 5/24
66			V												II			7m. 15d.	5/30 WC		POSIT			INFECTED	CALF DIED 5/30
126			V												II			7m. 15d.	6/3 WC		POSIT			INFECTED	CALF DIED 6/3
29			V												II			7m.	5/20 AB		POSIT	POSIT		INFECTED	
029			V												II			5m. 15d.	6/8 AB		POSIT	POSIT		INFECTED	
30			V												II			7m.	6/10 AB		POSIT	POSIT		INFECTED	
035			V												II			5m.	6/13 AB		POSIT			INFECTED	
55			V												II			6m.	6/9 AB		POSIT	POSIT		INFECTED	
064			V												II			6m. 15d.	5/29 AB		POSIT	POSIT		INFECTED	
068			V												II			6m 15d.	5/23 AB		POSIT	POSIT		INFECTED	
073			V												II			6m. 15d.	6/8 AB		POSIT			INFECTED	
075			V												II			7m.	6/16 AB		POSIT			INFECTED	
081			V												II			6m. 15d.	6/6 AB		POSIT	POSIT		INFECTED	
83			V												II			6m. 15d.	6/6 AB		POSIT	POSIT		INFECTED	
136			V												II			6m. 15d.	7/2 AB		POSIT	POSIT		INFECTED	
831			V												II			7m.	6/18 AB		POSIT			INFECTED	
970			V												II			5m.	7/16 AB		NEG		POSIT	INFECTED	
990			V												II			7m.	6/9 AB		POSIT	POSIT		INFECTED	

Group C^2 – Unvaccinated Controls
Pregnancy Time at Exposure 5m. 5d.-5m. 25d.

Nº	1951 2	3	4	5	6	7	8	9	10	11	12	1952 1	2	3	4	5/5	5/28	PREGNANCY TIME AT EXPOSURE	DATE NB - AB	POST PARTUM AGGLUT	BACTERIOL. INVEST. FETUS	PLACEN	COLOST.	IMMUNOLOGIC CLASSIFICATION	OBSERVATIONS
026															II			5m. 5d.	6/12 AB		POSIT	POSIT		INFECTED	
046															II			5m. 12d.	6/6 AB		POSIT	POSIT		INFECTED	
918															II			5m. 8d.	6/10 AB		POSIT			INFECTED	
950															II			5m. 12d.	5/21 AB		POSIT			INFECTED	
960															II			5m. 6d.	6/12 AB		POSIT			INFECTED	
963															II			5m. 6d.	6/12 AB		POSIT			INFECTED	
971															II			5m. 20d.	6/27 AB		POSIT	POSIT		INFECTED	
995															II			5m. 25d.	6/23 AB		POSIT			INFECTED	

= POSIT. AGGLUT. TITRE 1/100 OR HIGHER
= POSIT. AGGLUT. TITRE 1/50
= POSIT. AGGLUT. TITRE 1/25
= NEGATIVE AGGLUTINATION

V = vaccinated; ♀ = breeding time; II = 5×10^{-1} conjunctival exposure; NB = normal birth; AB = abortion; WC = weak calf.

and the bacteriological investigation proved negative, whereas the blood test revealed a titer of 1:100 or higher. Seven remaining cows (77.7 %) showed their infectious state as follows: 6 aborted with recovery of the organism; 1 gave birth to a weak calf which died three days later, being negative to the bacteriological investigation but showing a positive agglutination titer higher than 1:100.

The pregnancy stage at exposure of subgroup B-2 (strain B vaccine) ranged from five to seven and a half months (mean pregnancy stage 200 days). In this group, there

TABLE 3—Comparative Reactions of Vaccinated and Unvaccinated Pregnant Animals Exposed to Two Potencies (Infections I and II) of 5 Million and 50 Million Virulent Organisms—Groups A-1, A-2, B-1, B-2, C-1, and C-2

Animals Vaccinated with Strain 19(BAI).

		NB	WC	AB	BACT. INVEST.	IMMUNE	INFECTED	
							AB. RESIST.	WC - AB.
Group-A^1	INF. I 5×10^{-6}	3 = 50%	1 = 16.6%	2 = 33.3%	NEG 3, POS —, POS 3	3 = 50%	—	3 = 50%
Group-A^2	INF. II 5×10^{-7}	2 = 22.2%	1 = 11.1%	6 = 66.6%	NEG 1, POS 1, POS 7	1 = 11.1%	1 = 11.1%	7 = 77.7%

Animals Vaccinated with Strain "B"

		NB	WC	AB	BACT. INVEST.	IMMUNE	INFECTED	
							AB. RESIST.	WC - AB
Group-B^1	INF. I 5×10^{-6}	10 = 47.6%	2 = 9.5%	9 = 42.8%	NEG 4, POS 6, POS 11	4 = 19%	6 = 28.5%	11 = 52.3%
Group-B^2	INF. II 5×10^{-7}	3 = 14.2%	3 = 14.2%	15 = 71.4%	NEG 1, POS 2, POS 18	1 = 4.7%	2 = 9.5%	18 = 85.7%

Unvaccinated Controls

		NB	WC	AB	BACT. INVEST.	IMMUNE	INFECTED	
							AB. RESIST.	ABORTIONS
Group-C^1	INF. I 5×10^{-6}	2 = 18.1%	—	9 = 81.8%	NEG 2, POS —, POS 9	2 = 18.1%	—	9 = 81.8%
Group-C^2	INF. II 5×10^{-7}	—	—	8 = 100%	NEG —, POS —, POS 8	—	—	8 = 100%

NB = normal births; WC = weak calves; AB = abortion; INF. I = virulent exposure 5 million organisms; INF. II = virulent exposure 50 million organisms.

were three normal births (14.2 %), 3 weak calves which died soon afterward (14.2%), and fifteen abortions (71.4 %). Only 1 of the cows that calved normally proved negative both in the bacteriological and serological investigations, while the other 2 cows as well as those which gave birth to weak calves and those which aborted revealed Brucella infection. It is possible that cow 52 acquired its resistance prior to vaccination.

The animals of subgroup C-2 (unvaccinated controls) were exposed with pregnancies varying from five months and five days to five months and twenty-five days. All aborted and Brucella infection was demonstrated by recovery of the organism and general rising of agglutinin antibodies.

DISCUSSION

The standard of analysis used in the interpretation of the results of this experiment was chosen in accordance with the various angles suggested by the pathology of bovine brucellosis and their correlative immunity phenomena. Animals which, when facing Brucella exposure, calved normally and did not acquire infection as judged by recovery of the infective organism or by the presence of agglutinins at high titers (1 : 100 or above) were considered immune. Aborting animals, as well as those which produced weak calves, were considered infected as long as the virulent organism could be recovered or the rising of agglutinins was demonstrated.

Animals which calved normally, but were revealed to be infected by any of the aforementioned procedures were qualified as "infected abortion-resistant." This resistance to abortion, despite a remnant infection, may be important from the livestock production standpoint, whereas it is not important when considered in a rational plan for the eradication of the disease. So far, it lacks a definite immunological significance, as it cannot be stated whether it represents a special state of immunity with an undetermined mechanism or if it is related to a lower degree of infection which does not reach the abortion stage. The experimental results achieved in accordance with the previously mentioned standards of comparison are summarized in table 3.

This research has been performed under very severe test conditions, both from the standpoint of the doses and the virulence of the infective strain used, and by the strict methods of control applied. The division of each vaccinated group into two subgroups has reduced the significance of statistical values arising from the experimental re-

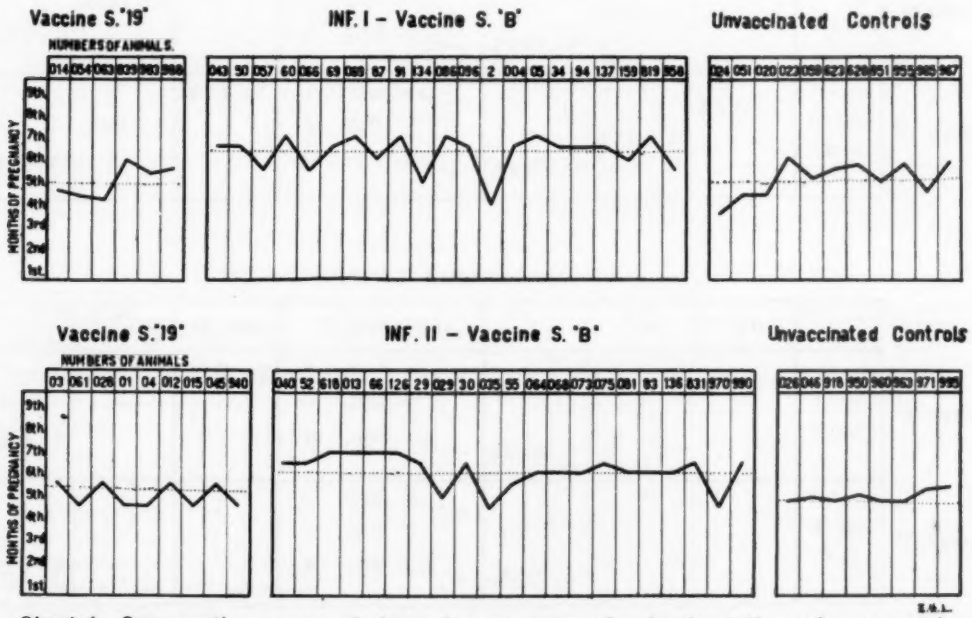


Chart 1—Comparative curves of stages in pregnancy of animals at time of exposure to virulent brucella organisms.

sults. This applies especially to the subgroups of animals exposed to the lower infective dose of 5 million organisms in which the comparative results showed a more noticeable difference in anti-infectious immunity. Infection II (50 million organisms) proved too strong, since it produced abortion in 100 per cent of the unvaccinated controls. However, both vaccinated groups gave better results than the controls.

In the two groups of animals vaccinated and exposed to the lower infective dose, it can be seen that, whereas the antiabortive protection values were 50.0 per cent for strain 19 (BAI) and 47.6 per cent for strain B, the anti-infectious immunity values were 50.0 per cent and 19.0 per cent respectively.

It should be noticed, however, that there is a differential factor in both vaccinated groups, which escaped our attention when the experiment was organized, *i.e.*, the difference in stages of pregnancy. In this respect, while the animals vaccinated with strain 19 were exposed at a mean pregnancy stage of 162 and 172 days, respectively, for subgroups A-1 and A-2, those vaccinated with strain B were infected at pregnancies averaging 202 and 200 days, respectively, for subgroups B-1 and B-2. This difference could be due to a greater maturity of the heifers selected for strain B vaccination, or because of some kind of mishandling of the bulls in the experimental farm. The difference in pregnancy stages is shown in chart 1.

The degree of susceptibility of the host toward Brucella invasion, at gestation periods ranging from five to seven months, is not well defined; yet, we have a certain number of observations indicating that the more advanced the pregnancy, the greater the susceptibility of the cow to infection. In a previous paper [5] dealing with a comparative immunity test in heifers vaccinated subcutaneously and intradermally with a strain 19 (BAI) vaccine, the experimental results also favored the group with the shortest pregnancy time. This observation has already been made by other authors. Beach, Irwin, and Berman [6] demonstrated that heifers, infected during the second and third months of gestation, were much more highly protected than those which received the virulent dose at pregnancies of four and a half to six months.

It is possible that the various organic phenomena which partake in the origin and development of the embryo may give rise to a state of increasing susceptibility as the gestation advances. On the other hand, as the fetus advances in age, there is an acceleration in its development and an activation of the physiological phenomena which intervene in its adaptation to independent life.

In the early periods of embryonic life, oxygenation of the tissues is made through the omphalomesenteric circulation. However, as the fetus develops this becomes less and less important, until it disappears and the hematosis is produced through the placental circulation, by gaseous interchange between the maternal placenta and the fetal chorion. Simultaneously, the maternal placenta acquires greater importance as a source of nutrition to the fetus, since it takes from the uterine circulation the nutritive substances which are absorbed by the epithelial villi of the fetal chorion opposite to the cotyledons. Thus, as gestation advances there is a greater dependence of the fetus on the maternal placenta. The latter, at this time, acts as a better field for the installation and progress of a Brucella infection, due to its greater development and functional activity.

It is understandable that if infection sets in under these conditions, the inflammatory and necrotic changes produced will affect in a greater degree the circulation and nutrition of the fetus, with greater possibilities of its death and premature expulsion. The different stages of pregnancy would consequently have a great influence on the nature, type, and intensity of the manifestations observed in the usual cases of field infection. These circumstances could account for the different results obtained on field vaccinations, which have sometimes proved surprisingly contradictory.

Under the experimental conditions of this research, both the group of animals vaccinated with strain 19 and those which received the nonagglutinogenic strain B demonstrated considerable antiabortive protection in relation to the unvaccinated controls. Admitting the strict experimental nature of the final results, which favor strain 19 vaccine to a certain degree, it is thought that the use of a nonagglutinogenic Brucella vaccine such as strain B is fully justified, since the nonproduction of agglu-

tinins permits its adaptation to the conditions under which it is necessary to act in the prophylaxis against bovine brucellosis.

This applies especially to those areas in which it is not possible to carry out prophylaxis based on the recognition, elimination, or segregation of carriers, which is a condition prevailing in most places. Furthermore, the combined use of both strains could be suggested in heavily infected premises, vaccinating the calves each year with strain 19 and revaccinating them with strain B. This procedure could be accompanied by the vaccination of all negative adult females with the same strain, irrespective of their age or whether they are pregnant or not, without the interference of postvaccinal agglutination titers.

A similar opinion has been expressed by Edginton and King [7] with respect to the M vaccine of Huddleson which produces short-lasting agglutinins, after a series of studies based on comparative tests between that strain and strain 19.

SUMMARY

Two groups of heifers 12- to 14-months old were vaccinated with vaccines prepared from strain 19 (BAI) and the nonagglutinogenic strain "B" (Rosenbusch), respectively. A third group of unvaccinated animals was included in the experiment. After service, each group was divided into two approximately equal subgroups, with each of them receiving a different infective dose. The antiabortive protection values for animals vaccinated with strain 19 was 50.0 per cent and 22.0 per cent, while for those vaccinated with strain B it was 47.6 and 14.2 per cent. The rate of anti-infectious immunity was 50.0 per cent and 11.0 per cent for strain 19, and 19.0 per cent and 4.7 per cent for strain B. The unvaccinated controls aborted at the rate of 81.8 per cent with the lower infective dose, while 100 per cent aborted with the higher dose.

It is thought that the animals vaccinated with strain B could have been at a disadvantage in relation to those of strain 19, as they were exposed at a more advanced state of pregnancy. Notwithstanding the apparently lower immunizing power of the nonagglutinogenic vaccine, it could be used since it does not interfere with the indispensable investigation of agglutinins in any complete plan of prophylaxis. This characteristic permits, in addition, the revaccination of heifers and the vaccination of adult females of any age and state of pregnancy. Finally, the use of both strains is suggested, reserving strain 19 for the primary vaccination of calves and using strain B for revaccinations and for vaccination of adult females.

References

[1] Rosenbusch, F.: Mem. Inst. Biol. Soc. Rural Argentina. (May, 1922): 23.

[2] Rosenbusch, C.: Prevención de la Brucelosis por medio de una Vacuna a Base de Cepas Atenuadas no Aglutinógenas (Cepas MBB). Mem. Primer Cong. Nacional de la Brucelosis del Uruguay. (Dec., 1947): 218-237.

[3] Sieiro, F., and Rosenbusch, C.: Comparative Immunity Experiments in Cattle Using Live Vaccines Prepared from Agglutinogenic and Nonagglutinogenic *Brucella Abortus* Strains. Am. J. Vet. Res., *13*, (1952): 476.

[4] De Mello, G. C., Danielson, J. S., and Kiser, J. S.: The Toxic Effect of Buffered Saline Solutions on the Viability of *Brucella Abortus*. J. Lab. and Clin. Med., *37*, (1951): 579.

[5] Sieiro, F.: Prueba Comparativa de Immunidad Antibrucélica en Bovinos con Vacunas a Cepa 19 por vías Intradérmica y Subcutánea. Gaz. Vet., *15*, (1953): 111.

[6] Beach, B. A., Irwin, M. R., and Berman, D. T.: Response of Previously Vaccinated Animals to Exposure in the Third Gestation Period with a Virulent Strain of *Brucella Abortus*. J.A.V.M.A., *110*, (1947): 355-361.

[7] Edginton, B. H., and King, N. B.: A Summary of Results Obtained in Ohio in Comparative Tests of Huddleson's Brucella Mucoid and Strain 19 Vaccines. Proc. U. S. Livestock San. A., (1952): 126.

Rumenitis and Its Relation to Rate of Change of Ration and the Proportion of Concentrate in the Ration of Cattle

RUE JENSEN, D.V.M., Ph.D.; W. E. CONNELL, M.S.; A. W. DEEM, D.V.M., M.S.

Fort Collins, Colorado

IN FATTENED beef cattle, rumenitis occurs commonly. The lesions probably lower efficiency of utilization of feed. Through foci of injury, bacteria, especially *Spherophorus necrophorus*, are able to penetrate the ruminal wall, enter the portal blood, and be carried to the liver where secondary foci of infection may become established. This study was made to determine the effect on the rumen of (1) rapid change of ration from roughage to concentrate and (2) the proportion of concentrate to roughage in the fattening ration.

REVIEW OF LITERATURE

Smith[2] studied rumens and livers of 1,807 fat cattle. The association of liver abscesses and ruminal injury was shown. Smith classified ruminal injuries as (1) adherent contents, (2) denuded, depigmented, and eroded areas, (3) ulcers having pseudodiphtheritic membrane, (4) clean ulcers, (5) scar, (6) papillomatous proliferations, (7) clumped villi, and (8) submucous nodules.

Jensen, Deane, Cooper, Miller, and Graham[1] studied stomachs and livers of 1,535 fat cattle and found high statistical significance for the concurrence of liver abscesses and gastric injuries. Ruminal injuries were classified as acute and chronic forms. Gross pathology and histopathology of the forms were described. Those authors showed that feeding large amounts of barley to cattle which were unaccustomed to grain in their ration produced lesions in the mucosa which were similar to lesions of spontaneous rumenitis of fat cattle.

From the School of Veterinary Medicine (Jensen and Deem) and the Department of Animal Husbandry (Connell), Colorado A. and M. College, Fort Collins.

This experiment was supported by the meat-packing industry of Denver, Colo., and by Livestock Conservation, Inc. Dr. J. W. Hovorka and his staff, Bureau of Animal Industry, Denver, assisted in the examination of organs of experimental animals at slaughter.

MATERIALS AND METHODS

Two experiments were conducted. The first was designed to determine (1) the effect on the rumen of rapid change of ration from roughage to concentrate, and (2) the ability of calcium carbonate, by neutralizing excess acidity, to protect against rumenitis. The second experiment was designed to determine the effect on the rumen of the ratio of concentrate to roughage in the fattening ration.

Experiment 1.—Cattle.—Ninety-five animals, 12 to 18 months of age, were arranged to form five equal lots. The majority of animals was Herefords. A few cattle of each lot were Aberdeen Angus, and Guernseys. Animals were weighed individually at 0, 72, and 133 days on fattening feed. Four animals from each of lots 1 through 4 were slaughtered after thirty-five days of fattening, while the remaining animals were slaughtered after 133 days of fattening. At the time of slaughter, stomachs were examined carefully for abnormalities. In case of rumenitis, the affected area was measured in square centimeters and located anatomically. All data were subjected to statistical analysis.

Feed.—All lots received a basal ration which consisted of chopped alfalfa, corn silage, corn, barley, linseed meal, mineral, and salt. A full

TABLE 1—The Effect on Rumenitis in Cattle of Rate of Change of Ration from Roughage to Concentrate

	Lots				
	1	2	3	4	5
Steers					
Fed 133 days					
Number	15	15	15	15	19
Initial wt. ave. (lb.)	591	582	584	600	580
Ave. daily gain/head (lb.)	2.63	2.70	2.58	2.45	2.93
Fed 35 days					
Number	4	4	4	4	
Days to full feed concentrate	12	30	12	30	12
Rumenitis					
CM^2 average/head	192.0	96.0	116.0	52.0	284.0
% in anterior ventral sac	91.0	100.0	89.0	85.0	92.0
Animals with rumenitis	12	9	12	13	12
Ration	basal	basal	basal + $CaCO_3$	basal + $CaCO_3$	basal

TABLE 2—The Effect on Rumenitis in Cattle of the Proportion of Concentrate to Roughage in the Ration

	Lots				
	7	8	9	10	11
Steers	6	8	7	8	8
Days of fattening	192	192	199	213	192
Rumenitis					
CM^2 average/head	195.0	4.25	14.3	0.5	16.8
% in anterior ventral sac	100.0	100.0	100.0	100.0	98.0
Concentrate: roughage	3:1	2:1	1:1	1:2	1:2 1st 28 days 1:1 2nd 28 days 2:1 3rd 28 days 3:1 last 108 days
Feed/head/day ave. (lb.)					
Corn, ground	7.41	6.73	5.30	3.63	6.52
Barley, rolled	7.41	6.73	5.30	3.63	6.52
Soybean meal	0.82	0.68	0.44	0.26	0.68
Alfalfa, chopped	4.94	6.73	10.77	14.47	7.03
Limestone, ground	0.42	0.34	0.18	....	0.32
Salt	0.05	0.06	0.08	0.07	0.07
Total	21.05	21.27	22.07	22.06	21.14

Fig. 1—Acute rumenitis in anterior ventral sac of animal after thirty-five days on fattening ration. Mucosa shows vesication, necrosis, and devillation. Arrows point to collapsed vesicles. A line encircles an area of necrosis.

Fig. 2—Acute rumenitis in anterior ventral sac of animal after thirty-five days on fattening ration. Mucosa shows necrosis of villi, hemorrhage, and beginning devillation.

daily feed of ration consisted of corn 8 lb., barley 4 lb., linseed meal 1 lb., alfalfa 4 lb., and corn silage 10 lb. Lots 1, 2, and 5 received basal ration only, while lots 3 and 4 received basal ration plus 1 lb. of calcium carbonate per head per day.

At the beginning of the experiment, all lots received alfalfa only. Gradually and continuously the proportion of concentrate was increased and the proportion of roughage was decreased. The daily ration was fed in equal portions morning and evening. Lots 1, 3, and 5 made the change from roughage to a full feed of fattening ration in twelve days, while lots 2 and 4 made transfer to the same fattening ration in 30 days.

Experiment 2.—Cattle.—Thirty-seven Hereford steers, 14 months of age, were arranged to form five lots, approximately equal. All lots were fed to the same degree of fatness. All cattle of a lot were slaughtered on the same date. At slaughter all stomachs were examined for abnormalities. Areas of rumenitis were measured in square centimeters and located anatomically. All data were subjected to statistical analysis.

Feed.—The basal ration consisted of corn and barley (equal parts), soybean meal, chopped alfalfa, limestone, and salt. The proportion of concentrates to roughage for the lots varied in order as follows: 3:1, 2:1, 1:1, 1:2, and a periodic step-by-step change in ratio from 1:2 to 3:1. Feed ingredients of each ration were mixed together and these mixtures were full-fed at all times. Tables 1 and 2 summarize procedure for experiments 1 and 2.

RESULTS

Table 1 presents the amount and anatomical location of rumenitis in animals of experiment 1. Table presents the same data for animals of experiment 2.

Analysis of variance applied to the experimental data showed that (1) In experiment 1, lots 1, 3, and 5, which transferred to full feed of concentrate in twelve days, developed significantly more rumenitis than

Fig. 3—Acute rumenitis in anterior ventral sac of animal, and gross cross section of wall, after thirty-five days on fattening ration. Submucosa is edematous and gaseous.

Fig. 4—Chronic rumenitis in anterior ventral sac of animal after 133 days on fattening ration. Mucosa shows pits from old vesicles, a scar, and devillation.

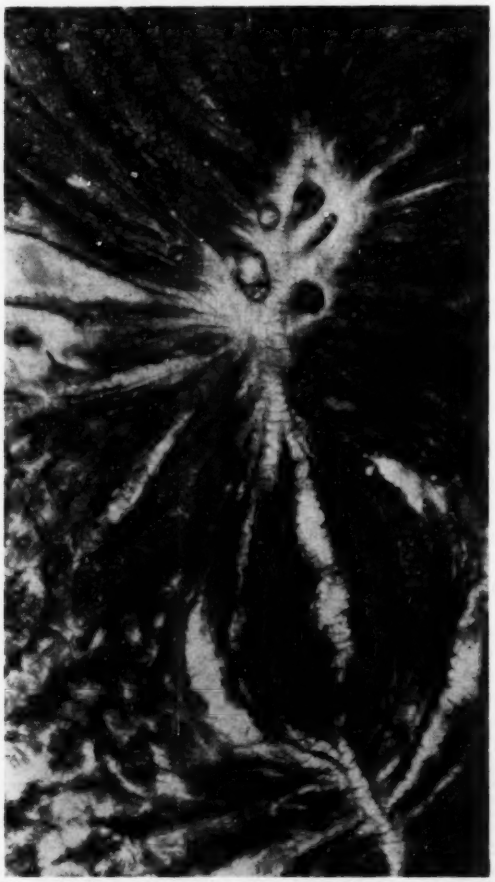


Fig. 5—Chronic rumenitis in anterior ventral sac of the type found in animals fed 192 days on a ration which consisted of 3 parts concentrate and 1 part roughage. Mucosa shows scars, devillation, and depigmentation. Villi are beginning to regenerate.

did lots 2 and 4 which transferred to the same ration of concentrate in thirty days; (2) in experiment 1, rumenitis in cattle of lots 3 and 4, which received calcium carbonate in their ration, was not significantly different in amount to rumenitis of cattle of lots 1, 2, and 5, which received no calcium carbonate; (3) in experiment 1, weight gains for the entire 133 days of fattening were not significantly different for the lots; (4) in experiment 2, lot 7 (ratio 3:1) developed significantly more rumenitis than did lots 8 through 11 which received the other ratios.

In animals slaughtered after thirty-five days of fattening, acute rumenitis was present. It was characterized by vesication, necrosis, devillation, hyperemia, and edema. In animals fattened 133 days, chronic rumenitis was shown. This was characterized by pits from collapsed vesicles, ulcers, scars, devillation, and depigmentation. Gross pathology is shown in figures 1 through 5.

The histopathology of acute and chronic rumenitis confirmed interpretations of gross changes. The gross pathology and histopathology have been described adequately by Jensen, Deane, Cooper, Miller, and Graham.[1]

DISCUSSION

Although not identified, the specific irritant which caused rumenitis was present in many animals in the anterior ventral sac during the first thirty-five days of fattening in experiment 1. The conditions which appeared to favor development of the irritant were (1) feeding a ration in which the ratio of concentrate to roughage was high and (2) rapid change from a ration of roughage to a ration high in concentrate.

SUMMARY

Two experiments were conducted to determine the effects on the rumen in cattle of the rate of change of ration and the ratio of concentrate to roughage in the ration.

Animals which transferred from a ration of roughage to a ration for fattening in twelve days developed significantly more rumenitis than animals which made the same transfer in thirty days. The sample of animals slaughtered after thirty-five days on fattening feed showed extensive acute rumenitis. Animals slaughtered after 133 days of fattening showed chronic rumenitis.

Cattle fattened on a ration in which the proportion of concentrate to roughage was 3:1 developed significantly more rumenitis than animals fattened on the same ingredients but in which the proportion of concentrate to roughage was 2:1 or lower.

References

[1] Jensen, Rue, Deane, H. M., Cooper, L. J., Miller, V. A., and Graham, R. W.: The Rumenitis-Liver Abscess Complex in Beef Cattle. Am. J. Vet. Res., *15*, (1954): 202-216.

[2] Jensen, Rue, Flint, J. C., and Griner, L. A.: Experimental Hepatic Necrobacillosis in Beef Cattle. Am. J. Vet. Res., *15*, (1954): 5-14.

[3] Smith, H. A.: Ulcerative Lesions of the Bovine Rumen and Their Possible Relation to Hepatic Abscesses. Am. J. Vet. Res., *5*, (1944): 234-243.

Experimental Infection of Calves with *Strongyloides Papillosus* (Nematoda)

HALSEY H. VEGORS, A.B.

Auburn, Alabama

Strongyloides papillosus, a rather small nematode parasite of the intestine of cattle and sheep, is cosmopolitan in distribution and of very common occurrence, especially in young animals. A previous report [1] gave its occurrence in about 21 per cent of the calves from southeastern states, but more recent observations in the aforementioned region show that about 80 per cent of calves under 4 months of age probably acquire this parasite.

Although the general features of the life history of *S. papillosus*, including the fact that infections can be established experimentally in rabbits either orally or percutaneously,[2] have long been known, existing reports do not show by which route the parasite is able to infect calves most successfully. Apparently, only one investigator has attempted to establish an experimental bovine infection,[3] using an ovine strain of the parasite. He did not demonstrate that the infection became patent.

Although most authors have considered *S. papillosus* a relatively harmless parasite, the limited field observations [4,5] concerning its effects on cattle and calves, together with the symptoms observed [3] in the only known case in which an infection was experimentally induced, suggest that it is potentially a serious pathogen.

To determine more conclusively and under controlled conditions whether infections with Strongyloides can materially affect the health of calves, and whether the bovine strain can infect these animals more readily through the percutaneous route than through the mouth, the following experiments were carried out at the Regional Animal Disease Research Laboratory, Auburn, Ala.

From the Regional Animal Disease Research Laboratory, Agricultural Research Service, U.S.D.A., Auburn, Ala.

The author is now at the U. S. Regional Animal Disease Research Laboratory Substation, Georgia Agricultural Experiment Station, Experiment, Ga.

The writer expresses his appreciation to Dr. Dale A. Porter for much helpful advice in carrying out this investigation, and to Mr. J. T. Lucker of the Zoological Division for assistance in preparation of the manuscript.

Materials and Methods

The test calves, purchased at birth from dairies, were raised parasite-free until experimentally exposed to infection and were subsequently kept under conditions precluding extraneous infection. They were fed milk from nipple buckets twice daily until they were about 4.5 months old; alfalfa hay and grain were placed before them when they were about a week old; this feed comprised their ration after weaning. Calves 1056 and 1058 were initially infected with larvae from feces obtained from naturally infected calves; all larvae used thereafter were either the first or a later generation of progeny of the adult worms which became established in these 2 calves.

The culture medium was a wet mixture of calf feces and animal charcoal. Cultures were kept at room temperature, and larvae were isolated from them by the Baermann isolation apparatus. All larvae used were freshly isolated when the cultures were about seven days old. The number of larvae was estimated by a dilution counting method. The hair was clipped from skin areas to which larvae were applied. To test the oral route of infection, the larvae usually were administered by squirting the suspension into the back of the mouth by means of a dissecting blowpipe attached to a 5-cc. rubber bulb, but occasionally the calf was given grain or feed moistened with the larval suspension.

The course of infection in each calf was followed by making fecal egg counts at daily intervals during the prepatent period, and at weekly intervals thereafter. Large numbers of eggs were counted by the dilution technique, and small numbers by the flotation technique described by Stoll.[6,7]

Packed red cell volume percentages were determined by the Wintrobe and Landsberg hematocrit method. All calves were weighed regularly, usually at weekly intervals, as long as there appeared to be a possibility of a weight change resulting from the infection.

The number of worms recovered at autopsy was estimated by examining aliquots of suspension of the small intestinal contents and washings. Complete examinations for evidence of gross pathological changes were made at necropsy.

Experimental Results

Relative Efficiency of Infection Routes.—When 2 young calves (1082 and 1084, table 1) of comparable ages were subjected to infection, 1 orally and the other percutane-

ously with approximately 1 million larvae each, the peak number of eggs subsequently observed on fecal examinations was about three and one half times greater and was reached much more quickly in the case of the percutaneously infected calf than in the 1 given larvae orally. When each of 4 calves, 3.5 to 4.5 months old, was similarly exposed to infection with 50,000 larvae, similar results were obtained, except that the averages of the peak egg counts for calves 1097 and 1098, exposed percutaneously, were about 115 times greater than those for calves 1094 and 1099 which were given larvae orally. Evidently, the percutaneous route of infection is the more successful avenue of infection.

Prepatent Period.—In instances in which it was determined, the prepatent period was nine to eleven days, usually ten days; apparently neither the mode of infection nor age of the host, within the range of one week to seven months, appreciably affected the length of the prepatent period.

Patent Period.—The maximum possible patent period following a single exposure to infection was not determined. Calf 1080, exposed to infection five times within a period of about four months, had eggs in its feces continually from the tenth day after the first exposure to the forty-second day after the fifth exposure, slightly in excess of five months. However, even a light earlier exposure evidently affected the length of the patent period of a test infection as shown by the following facts not given in table 1.

A 7-month-old calf which previously had harbored a light naturally acquired infection, as determined by the presence of small numbers of eggs in its feces over a period of five and one half months, and a calf of comparable age in whose feces eggs never had been found were each exposed to infection percutaneously with 1,333,000 and 332,000 larvae, given two days apart. The feces of the previously infected calf became negative for eggs thirty-four days after the last of the two exposures, whereas the feces of the previously uninfected calf did not become negative until the eighty-third day.

Age and Course of Infections.—That age did have an effect on the course of the infections is shown by the following facts. When calves 1081, 1085, and 1096, 5.5 months, 4.5 months, and 1-week-old, respectively, were initially exposed to infection percutaneously by a similarly large number of larvae from the same culture, the highest egg count was observed in the feces of the youngest one, the peak of egg production being attained thirteen days after exposure. In the other 2 calves, the peaks were reached much later, occurring on the fortieth day for calf 1081 and on the fifty-ninth day for calf 1085.

The 3 calves died after infection, but the youngest one died first and the oldest last, twenty-two and seventy-two days, respectively, after exposure. When the 24-hour fecal outputs of the youngest and oldest calves were collected on the twenty-first day after exposure and aliquots of each output were examined microscopically for worms, it was found that 750 and 2,298 worms, respectively, had been eliminated by these calves in the stated period.

Acquired Resistance.—The following facts are interpreted as evidence of the development of an acquired resistance to infection with *S. papillosus*. Fecal egg counts either remained virtually unchanged or declined when calf 1080 which previously had been twice exposed to infection was subjected to two further exposures, and when calf 1084 which for practical purposes had been exposed to infection only once previously was reexposed to infection twice. That this was actually an acquired and not an age immunity was indicated by a test in which 2, 7-month-old calves, 1 twice previously exposed to infection and the other unexposed, were subjected to infection by a like test number of larvae (table 1). The latter calf became infected, but the former did not become reinfected. All of the aforementioned calves (1080, 1084, 1058, and 1059) developed an immunity within two months that was sufficient to give nearly absolute protection against reinfection, despite exposure to extremely large numbers of larvae.

Calf 1082 was originally heavily exposed to infection. Forty-five days later it was placed in a pen where it was subjected to continuous infection from fecal accumulation. Forty-seven days after being placed in the pen and ninety-two days after the initial infection, the calf died. Samples of various tissues (myocardium, tongue, shoulder muscle, larynx, esophagus, brain, lung, and liver) were examined for larvae. A 100-Gm. samples of thigh muscle contained 6 larvae with precipitates around the

mouth and anus of each. One 26-Gm. sample of esophagus yielded a single unsheathed larva with no precipitates and all other samples examined were negative for larvae.

In a typical case, on first exposure of the skin to larvae, the calf showed very little evidence of irritation and the skin reaction was a mild erythema lasting for only a few hours. On second exposure, there was more evidence of irritation, with serum exudation and scab formation, the scabs persisting as granular elevated areas for about a week. On third exposure, there was marked evidence of irritation including frequent urination; prompt, marked, and prolonged edema of the skin area; prompt and prolonged exudation of blood and serum, apparently from minute abrasions produced by the larvae. Scab formation was delayed until the sixth day after exposure, and the scabs persisted for fifteen days. Apparently the sensitivity was generalized, since the degree of the reaction over old and new exposure sites was equal. However, marked reaction did not invariably follow each reexposure.

Clinical Effects.—Seven of the 16 calves exposed to infection showed marked clinical symptoms and died. The number of worms recovered from them at autopsy is shown in table 1. Clinical effects also were observed in calves 1080 and 1084, which were 2 of the remaining 9 calves which survived and were saved for use in other experiments. The most characteristic symptoms were intermittent diarrhea, the droppings in some cases containing mucus and blood, loss of appetite and condition, and retarded growth. Diarrhea usually occurred during the first and second weeks after exposure but as a rule, continued irregularly thereafter. The daily weight gains of the heavily infected calves were about 35 to 79 per cent less than those of uninfected controls.

A typical protocol was as follows: Calf 1082 had diarrhea and the feces were flecked with blood from the sixth to the eleventh day after exposure. Intermittent diarrhea occurred thereafter until the calf died about three months after exposure. At the end of the second month, the calf lost its appetite for grain and, as a consequence, lost condition and its growth was retarded. About two weeks later, its feces became chunky and of a plastic consistency and were often covered with mucus and blood. In the final two weeks before its death, its feces again became light-colored and mushy. Its postinfection weight gain was 15 lb., as compared to a gain of 70 lb. in the same period by its control, a 17-lb. loss of weight occurring in the last month of infection. Also, calf 1080, shortly after its third exposure weighed the same as its control, since the latter had lost some weight as a result of a slight coccidial infection; yet in the next five months, the control gained 66 lb. more than calf 1080. Calf 1081 which had gained well while serving as a control for calf 1080 for five and one half months previous to exposure (table 1) lost 10 lb. before it died, seventy-two days after exposure. The postinfection weight loss in calf 1085, which died fifty-nine days after exposure (table 1), was 52 lb., nearly a pound a day.

The calves most seriously or fatally affected were those which received a single heavy exposure to infection percutaneously. Among the older calves, the lowest exposure with such effect involved 1 million larvae in calf 1089, whereas among the very young calves such effect was produced by 200,000 larvae (calf 1090, table 1). The smallest number of worms recovered from a fatal case was 6,000 immature worms in a very young calf (calf 1111). In some instances, diarrhea and the peak of egg production occurred at about the same time.

No symptoms of pulmonary involvement were noted. The temperatures of 5 calves (3 exposed previously, 2 not exposed) were taken frequently during the first eleven to fourteen days after exposure to infection. The temperatures of those previously exposed were no higher than the others and none was above the normal maximum for cattle. The pulse rates of the first 2 calves, taken frequently during the first three days of the observational period, did not deviate from normal. No evidence that Strongyloides infections caused anemia was observed. Packed red cell volume in the blood of several of the calves was found to vary from about 31.5 to 35.0 per cent before infection and from about 29.0 to 34.0 per cent after infection.

Gross Pathology.—The most marked pathological changes found at necropsy were in the small intestine, especially in the duodenum and jejunum where the presence of the worms caused catarrhal inflammation, petechiae, and ecchymoses.

Notes selected from the protocols are as follows: The duodenum of calf 1085 was hemorrhagic throughout, especially in the first 3 ft. of its length; near the entrance of the common bile duct, the contents were of a gelatinous consistency. In parts of the duodenum, especially the posterior third, the lumen was greatly reduced as a result of catarrhal inflammation. The anterior extremity of the jejunum was erythematous and, in the posterior half, areas of the mucosa were edematous. Hard feces were

TABLE 1—Data on Experimental Infections with Strongyloides Papillosus in 16 Calves

Calf	Exposure age	Infective larvae given* (estimated)		Maximum e.p.g.** of feces	Interval between first exposure and maximum number of eggs in feces	Interval between last exposure and disappearance of eggs from feces	Days to necropsy		Worms recovered	Percentage of worms recovered in relation to number of larvae given
							From first infection	From last infection		
(No.)	(Days)	(Date)	(No.)	(No.)	(Days)	(Days)	(Days)	(Days)	(No.)	(%)
1056	115	6- 8-48	50,000(M)							
		7-23-48	8,100							
		7-27-48	6,000							
		8-10-48	3,000							
		8-12-48	8,000							
		8-17-48	4,500							
		8-24-48	30,000							
		9-21-48	333,333(M)							
			666,667							
		9-23-48	7,980,000(M)							
		9-30-48	2,330,000(F)							
		10- 9-48	2,000,000		21 and					
		10-26-48	155,000(M)	56	119	28	Survived		.....	.....
1058	109	6- 8-48	50,000							
		7- 7-48	96,000							
		9-21-48	333,333(M)							
			666,667							
		9-23-48	7,980,000(M)							
		9-30-48	2,330,000(F)							
		10- 9-48	2,000,000							
		10-26-48	155,000(M)							
		12- 2-48	1,330,000(M)							
		5- 4-49	4,450,000	1,280	21	0	Survived		.....	.....
1059	210	9-21-48	333,333(M)							
			666,666							
		9-23-48	7,980,000(M)							
		9-30-48	2,330,000(F)							
		10- 9-48	2,000,000							
		10-26-48	155,000(M)							
		12- 2-48	1,330,000(M)	60	21	0	Survived		.....	.....
1080	22	7-30-48	80,000							
		8- 3-48	60,000							
		8-17-48	170,000							
		10-28-48	50,000							
		11-29-48	500,000	199,200	32	42	Survived		.....	.....
1081	166	12-31-48	1,666,000	45,600	40	....	72	72	15,600	0.9
1082	10	8-15-48	550,000							
		8-16-48	500,000	343,200	18	....	92	91	48,500	4.6
1084	7	8-15-48	550,000(M)							
		8-16-48	500,000(M)							
		10-28-48	50,000							
		11-29-48	500,000	92,800	25	10	Survived		.....	.....
1085	143	12-31-48	1,666,000	90,400	59	....	59	59	192,000	11.5
1089	122	1-21-49	1,000,000	130,200	20	....	80	80	66,000	6.6
1090	19	11- 2-48	200,000	280,800	14	....	63	63	35,000	17.5
1094	131	4-25-49	50,000(M)	20	31	....	Survived		.....	.....
1096	7	12-31-48	1,666,000	235,600	13	....	22	22	307,500	18.4
1097	118	4-25-49	50,000	6,800	14	....	Survived		.....	.....
1098	111	4-25-49	50,000	2,400	14	....	Survived		.....	.....
1099	111	4-25-49	50,000(M)	60	21	....	Survived		.....	.....
1111	8	3-21-49	500,000	.....	....	....	7	7	6,000 Immature	1.2

* Given by skin unless indicated by M (mouth) or F (feed). ** e.p.g. = Eggs per gram.

present in the large intestine, apparently as a result of loss of muscle tone and consequent interference with peristalsis. This condition did not occur in the other calves. The abomasum was well filled and slightly impacted, the gall bladder enlarged, the bile was black in color, and the mesenteric lymph nodes were swollen and inflamed but lacked petechiae. The lymph nodes were normal in all other calves examined.

The small intestine of calf 1096 (table 1), which harbored more worms than any other calf examined, was inflamed and hemorrhagic throughout its length, especially in the duodenum and upper jejunum. Petechiae were present in the duodenum and ecchymoses were present in large areas of the jejunum, with a few of the latter in the pyloric region of the abomasum. Adult worms were found in tissue scrapings from these regions.

Edema and corrugation of the mucosa, as well as erythema, petechiae, and ecchymoses, were present in the anterior part of the intestine in calves 1111 and 1090. In calves 1111 and 1081, the first few inches of the cecum were inflamed.

SUMMARY

When calves were infected with *Strongyloides papillosus* larvae by mouth, the number of worm eggs subsequently eliminated with the droppings was practically negligible compared to those eliminated by calves infected percutaneously.

The prepatent period ranged from nine to eleven days, the worms becoming mature, as a rule, on the tenth day.

Calves infected when 4 to 5 months old survived infection longer than those infected at an earlier age. An acquired immunity was evinced by calves upon reexposure to larvae, but percutaneous reexposure produced a rather severe, localized inflammation in both old and new exposure sites.

The most frequently observed results of infection were intermittent diarhea, sometimes mucoid and bloody; loss of appetite; loss of condition; and retarded growth. Those calves severely infected made daily weight gains from 35 to 79 per cent less than those of comparable controls.

The worms produced a catarrhal inflammation with petechial and ecchymotic hemorrhages, especially in the duodenum and jejunum.

Experimental infections with *S. papillosus* killed 7 of the 16 calves. Very young calves were fatally affected by a single exposure of 200,000 larvae, while the least exposure associated with death of older calves was 1 million larvae.

References

[1] Porter, Dale A.: Incidence of Gastrointestinal Nematodes of Cattle in the Southeastern United States. Am. J. Vet. Res., *3*, (1942): 304-308.

[2] Ransom, Brayton H.: Notes on Parasitic Nematodes, Including Descriptions of New Genera and Species, and Observations on Life Histories. U.S.D.A. Circ., 116, (1907): 7 pp.

[3] Woodhouse, C. A.: Observations on Pathogenicity of Strongyloides Parasites in Ruminants. J.A.V.M.A., *113*, (1948): 354-356.

[4] Andrews, John S., Maldonado, J. F.: A Preliminary Note on the Internal Parasites of Puerto Rican Cattle with Special Reference to Those Species Found in Calves Suffering from "Tropical Diarrhea." J. Agric., Univ. Puerto Rico, *24*, (1940): 121-132.

[5] Roberts, F. H. S.: The Gastrointestinal Helminths of Cattle in Queensland: Their Distribution and Pathogenic Importance. Proc. Roy. Soc. Queensland, *50*, (1939): 46-54.

[6] Stoll, Norman R.: On Methods of Counting Nematode Ova in Sheep Dung. J. Parasitol., *22*, (1930): 116-136.

[7] Stoll, Norman R.: Observations on Cattle Nematode Infections, with a Demonstration of Their Secondary Transmission to Grazing Sheep. J. Parasitol., *22*, (1936): 386-407.

Application of the Capillary Tube Test and a Newly Developed Plate Test to the Serodiagnosis of Bovine Leptospirosis

HERBERT G. STOENNER, D.V.M.

Hamilton, Montana

IN A PREVIOUS publication,[1] the method of preparing antigens and the technique of conducting the capillary tube agglutination test for leptospirosis were described. Most of the serums used in the original evaluation and standardization of the test were free of hemoglobin and extensive contamination. With such serums, it was determined that the capillary tube technique and the agglutination-lysis method compared favorably both in sensitivity and specificity.

This paper deals primarily with the evaluation of the capillary tube method as a suitable test for the examination of cattle serums similar to those collected routinely in brucellosis control programs. Because such blood specimens are not collected in sterile tubes, many contain extensive bacterial growth and free hemoglobin upon arrival at the testing laboratory. Incidental to this study, data were collected on the prevalence of antibodies against *Leptospira pomona* among cattle in the Northwest. A rapid plate test, using the same antigen employed in the capillary tube test, is also described.

MATERIALS AND METHODS

Serums.—Serums utilized in the study had been submitted for tests for brucellosis to federal or state laboratories located in Oregon, Washington, Idaho, and Montana. After tests for brucellosis had been conducted, the clots were removed and the serums were forwarded for tests for leptospirosis. Because most of these specimens had been drawn six to ten days previously, many were hemolyzed or contaminated with bacteria. Such serums were centrifuged prior to testing. Others, however, were suitable for examination after erythrocytes had settled during overnight refrigeration.

Antigens.—Antigens used in this study were made from *L. pomona* (Johnson strain). The process employed was the same as previously described[1] with the following exceptions: (1) Cultures were regularly harvested after seven days of incubation and (2) the method was modified slightly to permit production of larger volumes of antigen and to reclaim a greater percentage of leptospiras previously lost during differential centrifugation. Centrifugations in an angle head at 3,000 g were conducted in 100-ml. heavy walled, round-bottom centrifuge tubes instead of 12-ml. conical tubes. Because the sedimented leptospiras are loosely packed, the supernatant fluid was carefully removed by aspiration through a capillary pipette attached to a vacuum system. In removing the leptospiras from the buffered 10 per cent formolized saline, the period of centrifugation was reduced from one and one-half hours to one and one-fourth hours. A few more leptospiras were left in suspension but the cells which were sedimented could be resuspended more completely. Centrifugation should be varied according to the length of the leptospiras, *i.e.*, strains of short leptospiras require a longer period of centrifugation and vice versa.*

After the leptospiras were centrifuged from the 10 per cent formalin, they were resuspended in a quantity of 15 per cent sodium chloride, borax-boric acid buffered solution equal to one tenth of the original volume of culture. If excellent growth had not been obtained, the organisms were resuspended in proportionately less solution. This suspension was then centrifuged for thirty minutes at 1,500 g in a horizontal head. The clarified suspension was decanted to clean 12-ml. conical centrifuge tubes. The sediment was resuspended in one half the volume of the previous centrifugation. This suspension was then similarly centrifuged for twenty minutes. The clarified supernatant fluid was again decanted, and the sediment was resuspended in one half the volume just previously centrifuged. After a similar centrifugation of this resuspension for twenty minutes, the clarified supernatant was decanted and the sedi-

This work was done at the U. S. Department of Health, Education, and Welfare, Public Health Service, Communicable Disease Center, Atlanta, Ga., and National Institutes of Health, National Microbiological Institute, Rocky Mountain Laboratory, Hamilton, Mont.

Dr. Stoenner's present address is National Institutes of Health, National Microbiological Institute, Rocky Mountain Laboratory, Hamilton, Mont.

Appreciation is expressed to Drs. H. F. Wilkins, A. E. Crouse, A. P. Schneider, and K. J. Peterson, state veterinarians, respectively, of Montana, Washington, Idaho, and Oregon for their cooperation in providing the bovine serums used in this study. The author is also indebted to Joseph Ayers for technical assistance in performing the tests.

* Recently, plate antigens were prepared from *Leptospira canicola*, *Leptospira icterohaemorrhagiae*, and *Leptospira hebdomadis*. It was observed that certain strains are more readily resuspended after fixation in buffered 10 per cent formolized saline of pH 8.5 (adjusted by varying the proportion of boric acid and boric solution).

ment was discarded. On each of the two ensuing days, the leptospiral suspension contained in 12-ml. conical centrifuge tubes was centrifuged in a horizontal head at 1,500 g for twenty minutes. After the last centrifugation, the supernatant fluid was decanted and pooled for standardization.

Certain serums escaped detection during screening although they were positive in high titer by the agglutination-lysis method. This was due to a prozone phenomenon. Such serums would agglutinate leptospiras on the plate-screen test if the antigen contained an adequate concentration of leptospiras. Therefore, the number of leptospiras in the plate antigen was increased so that all serums which were positive in high dilution would be detected during screening.

The density of plate antigens was standardized by a combination of colorimetric and serological methods. Each lot of antigen was tested against six or more serums which exhibited prozone properties. The antigen was adjusted until the following pattern was obtained with each of the serums:

Quantity of serum	*Undiluted antigen*	*50% antigen and 50% diluent*
0.005 ml.	4+agglutination	4+agglutination
0.01 ml.	4+agglutination	Negative to 1+ agglutination

It is apparent that the use of a properly standardized antigen would detect all serums with high titers, even if twice the prescribed amount of serum was used in the plate-screen test.

Leptospira pomona antigens, properly adjusted by serological means, gave 62 per cent light transmittance in a Klett Summerson photoelectric colorimeter equipped with a No. 42 filter. After it was determined that antigens standardized by serology gave consistent readings on the Klett colorimeter, lots of antigen were adjusted by colorimeter and then checked by the serological method. For use in the capillary tube test, 2 parts plate antigen were diluted with 1 part antigen diluent. To ensure uniformity, each lot of antigen was also tested by the capillary tube method against four pooled serums of known antibody content.

To evaluate the capillary tube technique, all serums originating from a herd in which one or more cattle serums reacted positively by the capillary tube method were also tested by the agglutination-lysis test. At the beginning of the survey, it was the intent to test serums from every infected herd by both methods. Laboratory facilities became overtaxed, however, by a rapid influx of serums after leptospirosis was associated with major outbreaks of abortions in beef cattle, and it was possible to perform duplicate tests on serums from only a limited number of herds. Agglutination-lysis tests were performed with the Johnson strain, following the method described in a previous publication.[1]

During the first part of the study, serums were screened by the capillary tube technique at 1:10 and 1:40 final dilutions. Those which caused significant agglutination of leptospiras at these dilutions were then retested in higher dilutions to determine their titer. During the latter part of the survey a macroscopic plate agglutination test for rapid screening was developed. The sensitivity of this test was standardized to detect serums varying in titer from 1:100 to 500,000 when tested by the microscopic agglutination-lysis method. Serums positive on the plate-screen test were tested by the capillary tube technique to ascertain their titer.

The following materials are required for conducting the rapid plate-screen test:

1) Two glass plates of appropriate size (10 by 12 in.).

2) Incubator box which can be placed over a shallow pan of water for incubating mixtures in a high relative humidity. Level supports are affixed to the inner sides of the box so that the plates can be placed on them through an open end.

3) Timer.

4) Loop standardized to deliver 0.005 ml. to 0.006 ml. of serum when touched to the surface of the glass plate. The amount of serum delivered by the loop should be checked occasionally, since certain serums that are positive in high titer exhibit prozone phenomenon if an excessive volume of serum is delivered. Appropriately graduated 0.2-ml. pipettes may be used in lieu of the loop.

5) Two beakers or flasks of water and a Bunsen flame.

TABLE 1—The Prevalence of Antibodies Against Leptospira Pomona Among Cattle in Montana, Idaho, Washington, and Oregon

State	Total herds tested	Total cattle tested	Negative herds Group A* Herds	Group A* Cattle	Group B** Herds	Group B** Cattle Pos.‡	Group B** Cattle Neg.	Herds with active infection † Herds	Cattle Pos.‡	Cattle Neg.
Montana	105	2,108	90	1,697	11	19	204	4	19	169
Idaho	142	886	103	411	25	42	217	14	122	94
Washington	55	1,483	36	179	7	15	188	12	455	646
Oregon	174	2,589	69	249	73	270	1,525	32	182	363
Totals	476	7,066	298	2,536	116	346	2,134	62	778	1,272

* No cattle with capillary tube serum titers of 1:40 or greater; ** one or more animals with capillary tube serum titers of 1:40 only; † herds which contain cattle with capillary tube serum titers of 1:160 or greater; ‡ includes animals reacting at 1:40 or greater on the capillary tube test.

6) Dropper calibrated to deliver 28 to 30 drops per milliliter of antigen. Antigen, not water or other liquids, should be used in calibrating the antigen dropper.

7) Applicator sticks or tooth picks for mixing serum and antigen.

8) Microscope lamp with clear glass filter or other suitable illumination for reading reactions.

9) Standardized leptospiral plate antigens.

The following procedure is used in screening serums by the plate method:

1) Deposit a loopful of clear undiluted serum on the plate. Wash loop in a beaker of water; flame loop and cool it in the second beaker of water. Loop is ready for use on the next sample after freeing it of excess water. No more than ten samples should be tested at one time.

2) Add a drop of antigen to the serum and mix each preparation with a fresh applicator stick. Spread drop to about 1/2-in. diameter so that drying around the edges does not occur during incubation.

3) To insure mixing, rotate plate five to six times.

4) Place plate in humidifier and incubate six minutes at room temperature. While this plate is incubating, ten serums can be prepared for incubation on the alternate plate.

5) Remove plate and rotate (tilt in circular motion) so that a portion of the mixture follows the entire periphery of the drop. The plate should be turned about 30 times per minute and should be tilted as far as possible without causing the drop to spread.

6) After a half minute of rotation, read and identify serums which show agglutination. These positive serums should be tested by the capillary tube test in final fourfold dilutions of 1:10 through 1:640 (1:5 serum dilution plus equal volume of antigen becomes a final dilution of 1:10). Rotate plate another half minute. Serums which become positive during the latter half minute are tested in final dilutions 1:10 and 1:40 by the capillary tube technique.

The reactions on the plate test are best read by examining the drops with transmitted light against a dark background. A direct beam of light from a Spencer-type microscope lamp (with clear glass filter) directed obliquely through the plate provides suitable illumination for reading the plate test. Agglutinated leptospiras appear first as clumps scattered throughout the drop. As the plate is turned, the clumps gather at the periphery so that the drop now consists of a ring of agglutinated leptospiras surrounding a clear area in the center. Bacterial and cellular debris usually gather in the central zone of the drop. A negative serum-antigen mixture is characterized by its homogeneous opalescence.

The titer of serums found to be positive in this screening procedure can be ascertained by a rapid plate method.[2] As fourfold serial dilutions of serum are prepared, 0.03 ml. of each dilution is deposited on a glass plate, and a drop of antigen is added to each serum dilution. After stirring, the mixtures are incubated for six minutes. The plate is then rotated for one minute and the reactions read. This method is useful if a prompt diagnosis is necessary, but it is not as adaptable to laboratory routine as the capillary tube method. The plate method also minimized cross reactions with other species of Leptospira. Titers of serums containing type-specific antibodies against the species incorporated in the antigen are usually equal by both techniques or may vary within one dilution of each other.

RESULTS

Serological evidence of infection with *L. pomona* was found among cattle in all four states surveyed. The data in table 1 do not reflect the actual incidence of the disease in these states, except possibly in Montana. Samples from this state were collected at random from lots of serums submitted during routine brucellosis testing. The survey began similarly in the

TABLE 2—Comparison of the Capillary Tube Technique and the Agglutination-Lysis Test in Examination of 504 Serums from Representative Herds Infected with Leptospira Pomona

Titer of serum examined by		Herds and number of cattle															
		A 127		B 16		C 18		D 49		E 159		F 101		G 34		Totals 504	
CT	ALT	CT	ALT	CT	ALT	CT	ALT	CT	ALT	CT	ALT	CT	ALT	CT	ALT	CT	ALT
Neg.	(1:10 or less)	119	119	14	14	16	16	35	37	21	19	35	25	8	8	248	238
1:40	1:100	2		2	2	1		7	3	18	7	49	31	4	0	83	43
	1:500		2				1		1		12		28		1		45
1:160	1:5000		3			1	1	3	4	35	16	9	7	3	4	51	35
	1:50,000		2						1		25		5		1		34
1:640 or >	1:500,000 or >	6	1					4	3	85	80	8	5	19	20	122	109
Percentage of correlation between tests *		100		100		100		95		99		84**		100		96	

A—undiagnosed calf losses; B—no symptoms; C—no symptoms; D—18% abortion rate, febrile anemia; E—25% abortion rate, no febrile anemia, *L. pomona* isolated; F—occasional abortions and calf losses; G—38% abortion rate, fatal febrile anemias.

CT—capillary tube test; ALT—Agglutination-lysis test; *—a titer of 1:40 or greater is considered a positive CT reaction, whereas a titer of 1:100 or greater is considered a positive AL reaction; **—all discrepancies at low titers.

other three states, but after it became apparent that serious outbreaks of abortion were associated with leptospirosis,* the method of collection of serums was changed so that specimens originated from enzootic areas. Also, many samples were submitted from brucellosis-free herds in which abortions had occurred. Therefore, the infection rate among herds in Idaho, Washington, and Oregon is much lower than the data in table 1 indicate.

TABLE 3—Comparison of Titers of 504 Bovine Serums when Tested Against Leptospira Pomona by the Agglutination-Lysis and Capillary-Tube Techniques

Titer with capillary-tube test	Titer with agglutination-lysis test					
	Neg. (1:10 or less)	1:100	1:500	1:5,000	1:50,000	1:500,000 or >
Neg. (1:10 or less)	233	13	2			
1:40	5	27	36	12	2	1
1:160		2	7	12	14	16
1:640 or >		1		11	18	92

Group B herds contained cattle whose serums gave positive reactions at dilutions of 1:40 or less when tested by the capillary tube method. In most of these herds, serums from only 1 or 2 animals in a herd reacted at this titer. It is thought that these low reactions reflect previous experience with *L. pomona*, cross reactions with other species of Leptospira, or cross reactions with other organisms. Because many of these herds were retested and no evidence of active intraherd dissemination was demonstrated, they were classed as negative herds.

Investigation of some of the reactors in group B herds revealed that they had originated from infected herds but had apparently ceased shedding leptospiras in their urine prior to their entry into the herd in question. In some group B herds, evidence that leptospirosis had occurred in the animals two to three years previously was obtained. Since many of the serums that reacted at 1:40 dilution on the capillary tube test originated from brucellosis-free cows which had aborted, an attempt was made to determine whether these reactions were specific or represented cross reactions with other organisms. A number of these serums were tested by the agglutination-lysis method against *Leptospira canicola, Leptospira icterohaemorrhagiae, Leptospira pomona, Leptospira hebdomadis,* and *Leptospira grippotyphosa.* Only irregular reactions in low dilutions of 1:10 and 1:100 were obtained. If abortions were attributable to one of the above species of Leptospira, one would expect much higher titers.

It is thought that some of the reactions reflect cross agglutination with *Vibrio fetus.* Serums from herds of cattle and sheep from which *V. fetus* was isolated reacted in low dilutions against *L. pomona* by both the capillary tube and agglutination-lysis techniques.[2] Such serums reacted in dilutions of 1:10 to 1:40 (occasionally 1:160) by the capillary tube test and 1:10 to 1:100 by the agglutination-lysis method. Although the possibility that these cattle and sheep had previously experienced contact with leptospiras can not be excluded, the results of these tests suggest that certain strains of *V. fetus* may possess antigens in common with *L. pomona.*

Serums from animals in representative herds infected with *L. pomona* were studied by the capillary tube and agglutination-lysis tests, and the results were compared. The percentage of correlation between techniques is presented because the reader can not compare results of tests of individual serums (table 2). It is evident that correlation between the two tests is excellent. In the case of herd F, similar results were obtained with both techniques in only 84 per cent of the serums. All discrepancies, however, were with serums which were either positive at 1:100 by agglutination-

* In the Northwest, leptospirosis occurs chiefly among herds of beef cattle. Abortion is a prominent symptom and in some herds as many as 35 to 40 per cent of pregnant animals abort. Many abortions occur during the last trimester of pregnancy, although some occur as early as the third month of gestation. A characteristic feature of the aborted fetus is an edematous umbilical cord which is usually severed close to the abdominal wall. Frequently, the placenta is firmly retained after abortion. Cases of febrile anemia and hemoglobinuria were observed among calves in infected herds.

lysis and negative by the capillary tube method, or positive at 1:40 dilution by the capillary tube technique and negative by agglutination-lysis. The majority of serums from animals in herds clinically affected by leptospirosis (D, E, and G) reacted in high titers by both techniques.

A comparison of titers obtained by examination of serums by the capillary tube and agglutination-lysis methods is shown in table 3. The titers of serums positive at 1:40 dilution by the capillary tube technique varied in the agglutination-lysis test, but the majority reacted in dilutions of 1:100 to 1:500. With serums having high titers in the capillary tube test, the correlation between titers obtained by both methods is closer. These data duplicate results obtained previously.[1]

In a preliminary report, it was stated that antigens remained stable for five to eleven months.[1] The stability of certain lots of antigen was again investigated. One hundred and fifty-two serums were tested with five different lots of antigen, and the results are presented in table 4. These data concern also the sensitivity of various lots of antigen. Results of these tests suggest that the antigen becomes slightly less sensitive with age. However, tests with lot 37 (19 months of age) compare favorably with other more recently prepared lots in reflecting the serology of these animals.

Additional data concerning the reproducibility of the results were obtained but are not presented in tabular form. It was found that 91 of these serums reacted to the same titer with each of the five antigens, 55 within one dilution, and only six gave titers that varied as much as two dilutions. One hundred and twenty-seven serums reacted to the same titer and 25 serums reacted within one dilution in tests performed with antigens 60, 65, and 72. Such variation is not solely a function of the sensitivity of the antigens but is also affected by technical error in diluting the serum and conducting the tests.

DISCUSSION

The combination of the plate-screen test and the capillary tube method provides a practical tool for the serodiagnosis of bovine leptospirosis. The screening technique enables one to rapidly classify serums into positive and negative categories. Because negative serums require no further handling after the screen test, a larger volume of samples can be tested by a combination of the plate and capillary tube methods than by any other technique available for leptospiral serology. This combination test, which is well adapted to laboratory routine, permits reporting of results within twenty-four hours after receipt of specimens.

The titer of serums found to be positive in the screen test can be determined by the capillary tube method or by the rapid plate technique. The former method has been found to be reliable in testing human, bovine, porcine, equine, and canine serums, but the rapid plate method is recommended for testing caprine serums. The capillary tube method is recommended for use in a laboratory supporting a mass-testing program. A larger volume of samples can be tested by the capillary tube method than by the plate method. In addition, antigens employed in the former test exhibit less species specificity, an attribute which is of value in the detection of antibodies against other species of Leptospira. The rapid plate method is adapted for use in the field and in situations where immediate results of tests are necessary. It would be particularly useful for the examination of cattle serums at public sale yards.

The proper interpretation of results of serological tests is important in the diagnosis and control of leptospirosis. Serums which agglutinate leptospiras at a 1:40

TABLE 4—Repeated Tests of 152 Cattle Serums from a Herd Infected with Leptospira Pomona to Demonstrate Uniformity and Stability of Antigen

Antigen lot no.	Preparation date	Date tested	Titers			
			Neg. (1:10 or less)	1:40	1:160	1:640 or >
37	1-13-52	8-18-53	26	28	29	69
52	3- 5-53	3-13-53	21	15	36	80
60	4- 7-53	8-18-53	17	21	31	83
65	4-27-53	8-18-53	18	21	28	85
72	7-15-53	8-18-53	16	19	21	96

dilution on the capillary tube test or at 1:100 by the agglutination-lysis method contain a low level of antibody against *L. pomona*. Possibly some of these reactions are nonspecific in nature. The diagnosis of an epizootic of leptospirosis, based solely on the demonstration of antibody in low titer in the serums of animals examined, is not justified. Cattle that have recently experienced leptospirosis usually possess antibody titers of 1:640 or greater when measured by the capillary tube method and corresponding titers of 1:50,000 to 1:500,000 on examination with the agglutination-lysis test. The stage of an epizootic can usually be determined by testing the serums of all animals within a herd and examining the distribution of the antibody titers obtained. In herds which have recently become infected, most of the serologically positive animals usually possess titers of 1:160 and greater, as shown in the capillary tube test. After infection has persisted in a herd for some time, the titers drop and the percentage of animals whose serums react at a 1:40 dilution thus increases. In herds in which cattle of all ages have continuous contact with each other, the presence or absence of infection among younger age groups is a factor in determining the time of the epizootic.

Considerable experience is required for the preparation of capillary tube antigens of uniform sensitivity and specificity. It is essential that leptospiras be cultivated in a medium which supports optimum growth since the organisms tend to clump and become elongated in a medium of improper composition. These tendencies drastically affect the yield and sensitivity of the antigen. Difficulties encountered in the cultivation of leptospiras may deter some laboratories from utilizing the capillary tube method for leptospiral serology. However, the antigens are sufficiently stable to make preparation and distribution commercially feasible.

Summary

A macroscopic plate-agglutination test, designed to enable easy and rapid classification of serums into positive and negative categories, is described. As evaluated through examination of 7,066 bovine serums, the combination of the plate test and capillary tube technique was found to be a practical serological tool. These serums originated from cattle in Montana, Oregon, Idaho, and Washington. Bovine leptospirosis was diagnosed in various counties of each state, but the disease was prevalent in circumscribed enzootic areas in Idaho, Oregon, and Washington. The specificity and sensitivity of the capillary tube method compared favorably with the agglutination-lysis test in duplicate tests of 504 serums originating from infected herds. Comparison of five lots of antigen of varying age indicated that these lots are reasonably uniform in sensitivity and that the antigen is stable.

References

[1] Stoenner, H. G.: A Capillary-Tube Test for Leptospirosis. Am. J. Hyg., 57, (1953): 316-327.
[2] Stoenner, H. G.: Unpublished data.

Studies on Neoascaris Vitulorum. II. The Resistance of the Ova to Certain Chemical Agents and Physical Factors Under Tropical Conditions

P. G. REFUERZO, D.V.M., and F. S. ALBIS-JIMENEZ, D.V.M.

Manila, Philippine Islands

THE PATHOGENICITY for the bovine species of *Neoascaris vitulorum* (Goeze, 1782) was previously pointed out by Vallisnieri and Descomps (cited by Neumann),[1] Kaupp,[2] Schwartz,[3] Neveu-Lemaire,[4] Monnig,[5] Morgan and Hawkins,[6] Refuerzo *et al.*[7] and others. Heavy infection causes unthriftiness, diarrhea, intestinal obstruction, and other disturbances that usually prove fatal. Due to the depredations of this worm, measures should be developed for its prevention and control. Accordingly, the resistance of the eggs was studied to increase the knowledge on the bionomics of the parasite.

PROCEDURE

A 3-week-old calf, heavily parasitized with this ascarid, at the Alabang Stock Farm, Rizal, P. I., served as the source of eggs during this study. Eggs in great number were obtained from the feces with the Kalantarjan's[8] flotation technique which employs sodium nitrate solution with a specific gravity of 1.40. The eggs were transferred to water in conical glass containers and washed several times by sedimentation to remove the salt and extraneous matter, then were dried for an hour in filter paper and divided into 10-mg. samples * by weighing on an analytical balance.

Group 1 consisted of these 10-mg. samples of "clean" eggs in the one-celled or two-celled stage. Group 2 consisted of similar samples of clean eggs in the embryonating (infective) stage. The samples in group 3 were different quantities of freshly voided feces containing an average of 321,000 eggs per gram.

The samples in groups 1 and 3 were immediately exposed to the factors or agents desired, while those of group 2 were first cultured to the infective stage in Petri dishes with little water at 27 C. for seventeen days.[7] The samples were then exposed to desiccation, to sunlight, or to sunlight with intermittent showers or rain, both with and without the protection of vegetation. They were also exposed to hot water and common disinfectants (lysol, creolin, and dyphen **). The effect of sunlight as a whole was studied and no attempt was made to differentiate the lethal action of its heat from its other property or properties.

Materials subjected to desiccation were placed in dry Petri dishes inside the laboratory at room temperatures of 27 C. to 29 C. The effects of sunlight either alone or with intermittent showers or rain (in the absence of vegetation) were determined by exposing the samples on a bare ground in the open. The exposure of such samples required meticulous care, in order that most of the eggs could be retrieved for examination. To determine the effects of hot water and chemical disinfectants on clean eggs, the samples were immersed in hot water of known volume and temperature or in disinfectants in the concentration usually employed. The same agents were poured on the fecal samples (group 3), placed on sandy loam soil, and examinations made when the agents dried.

To simulate pasture conditions under which feces of cattle are exposed to the elements, known quantities of stools with eggs (group 3) were placed under pasture grasses *(Pollinia fulva)* and legumes *(Pueraria javanica)* growing in plots in the Bureau compound at Pandacan, Manila, P. I. These forage crops were about 2 ft. tall and weighed 570 Gm. and 410 Gm., respectively, per square foot of growth at the termination of the experiment.

During this study temperatures and rainfall were recorded. For fecal samples, exposed directly to sunlight on bare ground or placed under forage crops, temperatures were taken twice daily (at 9:00 a.m. and at 3:00 p.m.) by putting the thermometer beside the samples for at least ten minutes. For clean eggs on the soil under direct sunlight, temperatures were taken as previously described every half hour. Room temperatures were read twice daily from the wall thermometers inside the laboratory.

The criterion for the efficiency of each factor or agent was its ability to destroy all the eggs

Dr. Refuerzo is Chief, Parasitic Diseases Division, Bureau of Animal Industry, Philippine Islands. Dr. Albis-Jimenez is veterinary helminthologist, Bureau of Animal Industry, Philippine Islands.

The authors express appreciation to Dr. Wilford S. Bailey, head of the Department of Pathology and Parasitology, School of Veterinary Medicine, Alabama Polytechnic Institute, Auburn, for his encouragement and for the many helpful suggestions and constructive criticisms without which this paper would not have been possible.

* The number of ova in each 10-mg. sample has not been definitely determined. However, counts of three samples gave an average of 2,836,000 eggs.

** Dyphen (Bairds) consists of alcohol (20 % by volume when formulated), 15 per cent by weight; orthophenylphenol and 2 chloro-4 phenylphenol, 7 per cent; soap, 10 per cent; inert ingredients, 68 per cent; and water.

in a given sample after a certain period of time as observed on periodic microscopic examination. For the nonembryonating eggs, the indexes of destruction were the failure of the eggs to continue development on culture and the vacuolation of their cellular contents. For the embryonating eggs, three conditions had to be satisfied, namely, (1) the cessation of movements of the embryos, (2) evidence of degenerative changes, and (3) their inability to infect susceptible laboratory animals.

Unless otherwise stated, at least three samples were provided for each exposure of the three groups, and the efficiency of the agent or factor involved was judged from the minimum length of exposure that destroyed all the eggs retrieved in all three samples taken together. Parallel control samples were provided in each case (table 1).

Discussion

The results indicate that the eggs of *N. vitulorum* have low resistance to the effects of direct tropical sunlight. Nonembryonating clean eggs were destroyed in five hours and thirty-five minutes when exposed directly to the sun on a bare, dry ground with temperatures between 34 C. and 42 C. (ave. 40 C.). On the other hand, most of

TABLE 1—Showing the Resistance of Neoascaris Vitulorum Ova to Certain Physical Factors and Chemical Agents

Samples							
Amount	No. exposed	Factor or agent	Temperature	Length exposure	Rainfall (mm.)	Effects on ova	Controls (condition of untreated ova)
			GROUP 1—"CLEAN" NONEMBRYONATING OVA				
10 mg.*	3	Sunlight, direct	40 C. ave. (34-42 C.)	5.6 hr.		Destroyed; vacuolated	96 % developed on culture
10 mg.	3	Desiccation (room)	27-29 C.	23 days		Destroyed; vacuolated	96 % developed on culture
			GROUP 2—"CLEAN" EMBRYONATING OVA				
10 mg.	3	Sunlight, direct	40 C. ave.	5.6 hr.		Most still viable	In group 2,
10 mg.	3	Sunlight, direct	42 C. ave.	1 hr.		Some still viable	most of the ova
10 mg.	3	Sunlight, direct	43 C. ave.	3 hr.		Destroyed; embryos degenerated	were viable
10 mg.	3	Sunlight, direct	48 C. ave.	2.5 hr.		Destroyed	
10 mg.	3	Hot water	92-100 C.	2 sec.		Destroyed	
10 mg.	3	Lysol (2 % solution)	29 C.	17 hr.		Destroyed	
10 mg.	3	Dyphen (4.5 % solution)	29 C.	17 hr.		Destroyed	
10 mg.	3	Creolin (2 % solution)	29 C.	20 hr.		Destroyed	
			GROUP 3—FRESH DEJECTA †				
62 Gm.	1	Sunlight, direct	36-45 C.	8 days		Destroyed; vacuolated	In group 3, most of the ova developed on culture
28 Gm.	3	Sunlight with showers (bare ground)	39 C. ave. (31-45 C.)	12 days	0.18 ave. (2.10 total)	Nonviable; stool reduced to 16 Gm. by close of experiment	
33 Gm.	3		34 C. ave. (29-45 C.)	12 days	0.41 ave. (5.0 total)	Destroyed; feces reduced to 21 Gm.	
36 Gm.	3		39 C. ave. (31-45 C.)	12 days	0.18 ave. (2.10 total)	Destroyed; feces reduced to 23 Gm.	
37 Gm.	3		33 C. ave. (29-37 C.)	25 days	0.68 ave. (17.10 total)	Destroyed; (cloudy with showers)	
50 Gm.	3		29 C. ave. (25-35 C.)	35 days	2.06 ave. (72.2 total)	Destroyed; (cloudy with showers)	
45 Gm.	3	Sunlight with showers under grass (*Pollinia fulva*)	30 C. ave. (24-35 C.)	66 days	1.26 ave. (83.0 total)	Destroyed; (cloudy with showers); feces reduced to 21 Gm.	
45 Gm.	3	Sunlight with showers under legume (*Pueraria javanica*)	29 C. ave. (24-35 C.)	76 days	1.09 ave. (83.0 total)	Destroyed; (cloudy with showers); feces reduced to 20 Gm.	
12 Gm.	3	Desiccation (room)	27-29 C.	33 days		All destroyed	
18 Gm.	3	Desiccation (room)	27-29 C.	35 days		All destroyed	
20 Gm.	3	Desiccation (room)	27-29 C.	39 days		All destroyed	
38 Gm.	3	Hot water	92 C.	Until dried		Only 8 % destroyed	
38 Gm.	3	Lysol (2 % solution)	29 C.	Until dried		Still viable	
38 Gm.	3	Dyphen (4.5 % solution)	29 C.	Until dried		Still viable	

* Number of ova not definitely determined but counts of three samples gave 2,836,000 eggs (ave.). † Eggs per gram, 321,000.

the infective ova were still viable after exposure for five hours and thirty-five minutes at an average temperature of 40 C. This indicates that the embryonating eggs are comparatively more resistant to direct solar effect than the nonembryonating eggs. According to Spindler,[9] the corresponding eggs of *Ascaris suis* exhibit a similar relative resistance when exposed to sunlight in the tropics. Direct exposures to sunlight at higher temperatures of 43 C. and 48 C. were lethal to the infective Neoascaris ova in only three hours and two and one-half hours, respectively. Working in the Philippines, Schwartz[10] also found the embryonating eggs of this cattle ascarid destroyed following their exposure for one hour at 45 C.

Eggs within the stool samples were able to survive longer periods of exposure to direct sunlight, the time depending on the temperature and on the size of the protective fecal mass. In a 62-Gm. fresh sample of stool (the full amount voided in one defecation by the calf), the eggs were nonviable after exposure for eight days at average daily soil temperatures of 36 C. to 45 C.

When exposed to sunlight with intermittent rains or showers, the time required to destroy eggs within the stool was much longer than when exposed to direct sunlight alone, due ostensibly to the lower temperature and to the presence of a greater amount of moisture. Ova in fecal lumps of 28 to 36 Gm. were destroyed after twelve days at temperatures of 34 C. to 39 C. and an average daily rainfall of 0.18 mm. to 0.41 mm. (total, 2.10 mm. to 5.0 mm. during this phase of the work). However, on days which were mostly cloudy with intermitent rains (and this frequently happens during certain months of the year) with average temperature of 29 C. (25 to 35 C.) and an average daily rainfall of 2.06 mm. (total, 72.2 mm. for the period embraced by this phase of the study), the ova in fecal lumps of 50 Gm. were only destroyed after a much longer period of exposure (35 days).

The eggs appeared resistant to desiccation. Kept at room temperatures of 27 C. to 29 C., the clean non-embryonating eggs lost their vitality and were completely destroyed after twenty-three days, while the eggs in fecal lumps of 12 Gm. to 20 Gm. were nonviable after thirty-three to thirty-nine days. (Schwartz[10] found the embryonating eggs of this nematode resistant to drying.)

While hot water of 92 C. or over destroyed the clean eggs almost instantaneously, it was only partially effective when applied to fecal lumps on sandy loam soil in amounts just enough to soak them. Only the eggs on the surface of 38-Gm. fresh stools were destroyed when the water cooled off and sank in the ground. Hot water can probably be utilized to greater advantage by using it for the disinfection of calf stalls with concrete floors where the agent can be held for a longer period to enable the heat to penetrate the fecal lumps in their entirety; otherwise, it has to be applied in bigger volumes.

Like those of *Ascaris lumbricoides*, the eggs of *N. vitulorum* are resistant to temporary baths in chemical disinfectants. Lysol, creolin, and dyphen, in their usual concentrations for disinfection, destroyed only the clean embryonating Neoascaris ova following their continuous immersion for seventeen to twenty hours, and had no lethal effect when poured on the dejecta with eggs on the soil. What factors or peculiarities of the ova are responsible for their resistance have not been studied, but they are probably not unlike those operating in *A. lumbricoides*. Citing Zawadowsky, Belding[11] attributes the resistance to chemicals of the eggs of the latter worm to the impermeability of the inner fibrous membrane, and adds that the outer covering of the shell affords protection against physical disturbances.

Placed under a fairly good stand of pasture grasses and legumes, as previously described, the eggs in 45-Gm. fecal samples lost their vitality and were innocuous, after sixty-six to seventy-six days of exposure, to the effects of intermittent sunlight and rain (table 1). This and the other findings may have some practical value for cattlemen, especially in the Philippines where neoascariasis is responsible for low calf crops on certain farms.[7] For purposes of pasture rotation, the findings indicate how long cattle pastures have to be vacated, in order to destroy Neoascaris ova, before they can be used again for grazing. From the results obtained, pastures may be rotated every three to four months in the

Philippines, or in other countries where conditions are similar.

SUMMARY

The resistance of *Neoascaris vitulorum* eggs to certain chemical agents and physical factors under tropical conditions was studied. The "clean" eggs had low resistance to the effects of direct tropical sunlight; the nonembryonating ova have a lower resistance than the embryonating ova. However, ova protected by the stool were able to survive longer periods of exposure to sunlight, the time varying inversely with the temperature but directly with the size of the fecal mass, the amount of rainfall, and the amount of vegetation. The ova were resistant to desiccation and to the usual concentrations of chemical disinfectants (lysol, creolin, and dyphen). While hot water was highly lethal to clean eggs or egg masses devoid of extraneous matter, it was only partially effective when applied to eggs within fecal lumps. The temperatures and periods of exposure to the different agents, that proved destructive to the eggs, are given. Suggestion on pasture rotation is included.

References

[1] Neumann, L. G.: A Treatise on the Parasites and Parasitic Diseases of the Domesticated Animals (Translated by George Fleming). 2nd ed. (revised and edited by James MacQueen). Bailliere Tindall and Cox, London. 1905.

[2] Kaupp, B. F.: Animal Parasites and Parasitic Diseases. 4th ed. Alexander Eger, Inc., Chicago. 1925.

[3] Schwartz, B.: Effects of Extracts of *Ascaris Vitulorum* on Experimental Animals. Philippine J. Sci., *22*, (1923): 109-114.

[4] Neveu-Lemaire, M.: Traite D'Helminthologie Medicale et Veterinaire. Vigot Freres, Paris. 1936.

[5] Monnig, H. O.: Veterinary Helminthology and Entomology. 3rd ed. William Wood and Co., Baltimore. 1949.

[6] Morgan, B. B., and Hawkins, P. A.: Veterinary Helminthology. Burgess Publishing Co., Minneapolis. 1953.

[7] Refuerzo, P. G., Albis-Jimenez, F. S., and Cerna, G. de la: Studies on *Neoascaris Vitulorum* (Goeze, 1782). I. Observations on the Morphology of the Ova and on Their Development to the Infective Stage. With Notes on Feeding Experiments. Philippine J. Anim. Indust. (in press).

[8] Kalantarjan, E. V.: Utilization du nitrate de sodium dans la pratique helminthologique. Med. Parasit. and Parasit. Dis., 7, (1938): 142-143. (In Russian, French summary).

[9] Spindler, L. A.: Effect of Tropical Sunlight on Eggs of *Ascaris Suis* (Nematoda), the Large Intestinal Roundworm of Swine. J. Parasitol., *26*, (1940): 323-331.

[10] Schwartz, B.: Observations on the Life History of *Ascaris Vitulorum*, a Parasite of Bovines in the Philippine Islands. Preliminary paper. Philippine J. Sci., *20*, (1922): 663-669.

[11] Belding, D. L.: Textbook of Clinical Parasitology Including Laboratory Identification and Technic. D. Appleton-Century Co., Inc., New York. 1942.

The Liver of the Camel as Revealed by Macroscopic and Microscopic Examinations

ABD EL HAMID HEGAZI, M.V.Sc.

Giza, Egypt

THE GREAT variation of the liver of the camel from that of other domesticated animals has encouraged me to briefly describe this organ from the anatomical, comparative, and microscopical point of view.

LITERATURE CITED

Chauveau [1] mentions that the liver of the camel has the same arrangement as in other ruminants except that its caudad face is divided, lobulated, and much reticulated. Lesbre [2] observed that the liver of the camel is on the right half of the abdominal cavity, as in other ruminants, and that it is divided laterally into three main lobes and other lobules. He also states that the most remarkable features of the liver are the absence of a gall bladder and the fissured appearance of its caudad surface. Leese [3] states that the liver of the camel is much fissured on the lower border of its caudad surface. He also mentions its resemblance to the liver of the pig in that the lobulations show clearly on the surface of the organ.

MATERIALS AND METHOD

The livers of 40 camels, slaughtered in abattoirs in Belbeis and Cairo, were subjected to detailed measurement and dissection. Other camels were dissected in the Anatomy Department after injecting them in the usual manner with preservative solution.

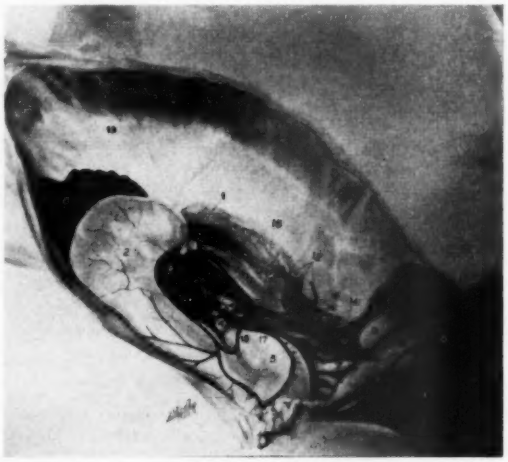
RESULTS

Macroscopic Examination.—Examination reveals that the liver of the camel is a darker brown and has a firmer consistency than that of other domesticated animals. It is completely situated on the right half of the abdominal cavity. The lobes, three in number, are not clearly defined, being marked only by faint fissures on the free lateral border of the liver. The superficial lobulation of the organ is well marked due to the excess of the interlobular connective tissue. In this feature it resembles, to some extent, the liver of the pig. The general shape of the liver of the camel is triangular, with the apex situated cranially. The liver of the adult camel measures about 70 cm. in length and 35 cm. in width (fig. 1), and its weight varies from 15 to 20 lb. and may reach 25 lb.

The parietal surface of the liver is convex, smooth, and is related to the diaphragm and the vertebral parts of the sixth to the eleventh ribs.

The visceral surface is very irregular in conformity with the organs related to it, the rumen, reticulum, omasum, abomasum, and pancreas. In addition, it presents numerous small lobules separated from each other by distinct fissures. The duodenum leaves a shallow impression of its S-shaped portion on this surface.

The portal fissure forms a well-defined depression located on the visceral surface close to the base of the liver and under cover of its middle lobe. The fissure contains the portal vein, hepatic vessels, and nerves. Numerous large hepatic lymph glands are grouped around its dorsal part,



(1) Esophagus; (2) omasum; (3) abomasum; (4) duodenum; (5) small intestines; (6) colon (large caliber); (7) colon (small caliber); (8) liver; (9) pancreas; (10) spleen; (11) kidney; (12) ruminal branches; (13) celiac axis; (14) splenic artery; (15) hepatic artery; (16) anterior mesenteric; (17) superior branch; (18) inferior branch; (19) diaphragm.

Fig. 1—Ventral view of the abdominal cavity of the camel showing the relative position of certain organs.

Dr. Hegazi is lecturer in anatomy, Faculty of Veterinary Medicine, Cairo University, Giza.

The author is grateful for the help given by Dr. El Hagri, professor of anatomy, in preparing and writing this article.

and the head of the pancreas is attached there.

The apex of the liver lies above the xyphoid cartilage of the sternum. It is markedly convex and irregular due to the presence of a large number of indentations.

The base is short, thick, and concave and presents, on its medial part, a small but deep impression which receives the anterior pole of the right kidney; on its lateral part, it presents a large but shallow impression for both the cranial extremity of the abomasum and the beginning of the duodenum. The caudal vena cava enters the base of the liver immediately dorsal to the renal impression.

The lateral border is remarkably thin and irregular due to the presence of numerous fissures of variable depth which partly divide the organ into faint lobes. This border extends from the right sixth intercostal space to the flank region on a level with the junction of the vertebral third and sternal two thirds of the ribs.

The medial border is thick and nearly straight and is mesially situated, lying ventral to the bodies of the thoracic vertebrae. This border also shows a deep full-length groove in which the caudal vena cava is lodged.

The liver of the camel is fixed in position by the right lateral ligament which attaches it to the anterior part of the sublumbar region, but the left lateral ligament is absent. The falciform ligament is present but not well developed. The lesser omentum is attached to the liver along a line extending from the esophageal notch to the portal fissure.

The hepatic duct is formed by the union of primary branches of the left and right lobar ducts. It measures about 3 in. in length and 1 in. in diameter and is joined by the pancreatic duct before it reaches the duodenum. The common, thin-walled, wide duct is brown and opens about 53 cm. caudad to the pyloric extremity of the abomasum.

Of special interest is the absence of a gall bladder, as is the case in the horse.

Microscopic Examination.—Unlike that of most domestic animals, the liver of the camel is characterized by an abundance of connective tissue (fig. 2). The hepatic lobules are distinctly separated from each other by thick strands of connective tissue which envelop the whole lobule. Some strands of connective tissue fibers intervene between the hepatic cells as pericellular connective tissue. The outer Glisson's capsule is somewhat thin, especially when compared to that of the ox. The liver of the ox has a somewhat thick Glisson's capsule, but the lobules lack the large amount of connective tissue present in the liver of the camel. In the ox, there is no distinct outline for the hepatic lobules which appear mingled with only a few connective tissue fibers at the angles of the lobules. The liver tissue of the ox is also more vascular than that of the camel.

By comparing different sections of livers of the camel and of the pig, it is clear that the liver tissue of the camel is richer in both interlobular and intralobular connective tissue than is that of the pig which, of the domesticated animals, has been considered to have the greatest amount of interlobular connective tissue.

Fig. 2—Low-power magnification of the camel s liver showing the heavy interlobular connective tissue.

CONCLUSION

The liver of the camel resembles that of other ruminants in being entirely situated on the right side and consisting chiefly of one lobe. It resembles that of the horse in the absence of the gall bladder and in being divided, faintly, into three lobes. It resembles that of the pig in the abundance of interlobular connective tissue.

It is also characterized by the lobulation of its caudal surface, the hard consistency of its tissue, and its dark brown color.

References

[1] Chauveau, A.: The Comparative Anatomy of the Domesticated Animals. W. R. Jenkins, New York, 1891.

[2] Lesbre, F. K.: Recherche Anatomique Sur Les Camélides. J. B. Baillière et Fils, Paris, 1903.

[3] Leese, A. S.: A Treatise on the One-Humped Camel in Health and Disease. Haynes and Son, Stamford, England, 1927.

A Brief Histology of the Intestine of the Turkey Poult

DONALD D. DEMKE, D.V.M., M.S.

College Station, Texas

WHILE ENGAGED in research on coccidiosis in turkey poults, no integrated or complete description of the normal histology of the turkey intestine could be found upon which to base pathological findings. The only comparable piece of literature found was by Calhoun [2] in which she described the histology of the intestinal tract of the domestic chicken *(Gallus domesticus)*. With this lack of information in mind, a brief description of the normal histology of the intestinal tract of turkey poults is herewith presented.

MATERIALS AND METHODS

Histological sections were made from the intestines of 16 Beltsville Small White poults ranging from 1 day to 12 weeks of age.

Portions were taken of the duodenum, upper intestine midway between the duodenum and yolk stalk, lower intestine midway between the yolk stalk and the opening of the cecums, middle portion of the cecums, and of the rectum between the cecal openings and the cloaca. These portions, as removed, were equally divided and one part was placed in 10 per cent formalin, the other in Zenker's fluid.

Paraffin sections cut at 7 μ were routinely stained with Harris' hematoxylin and eosin.[5] Special staining was done with Masson's trichrome stain for connective tissue,[3] Masson's section method for argentaffin cells,[4] and the DeCastro-Cajal method for peripheral nerves.

Approximately 1,300 sections were made with the following distribution: duodenum, 239; upper intestine, 127; lower intestine, 172; cecums, 472; and rectum, 331 sections.

DESCRIPTION

General.—In those portions of the intestinal tract from which sections were made and examined, no marked distinction could be seen between the parts except for the duodenum, cecum, and rectum.

Duodenum.—The tallest villi found in the intestinal tract were in this region. Many of the villi were branching. All were covered with simple columnar epithelium with a pronounced cuticular border. Many goblet cells were present.[1, 2, 6, 7] Crypts of Lieberkühn were very prominent. Argentaffin cells were found in the epithelium and tunica propria (fig. 1).

The tunica propria, extending into the villi and surrounding the glands of Lieberkühn, was very thin and was not differentiated into stratum compactum and stratum granulosum.

The submucosa was poorly developed. No Brunner's glands were noted in the sections examined.

The muscularis mucosae was well de-

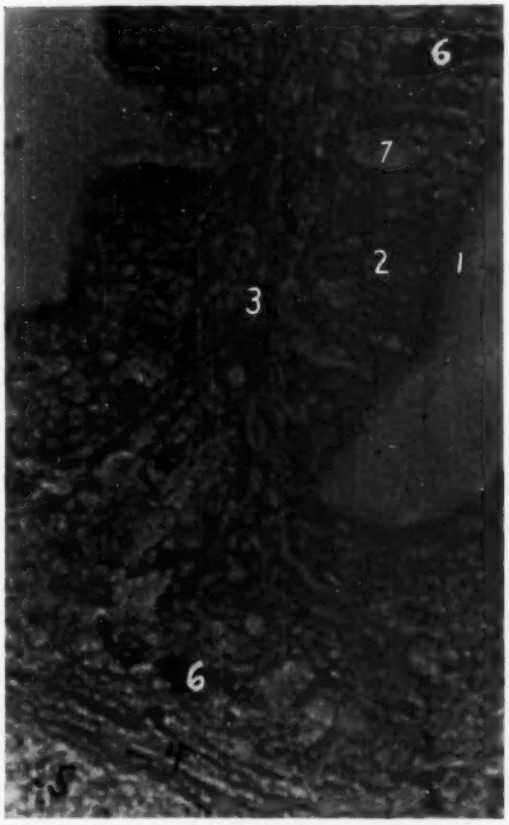


Fig. 1—Section of the duodenum of a turkey poult: (1) cuticular border, (2) epithelium, (3) tunica propria, (4) muscularis mucosae, (5) lamina muscularis (circular layer), (6) argentaffin cells, and (7) goblet cell. x 174.

From a portion of a thesis submitted as partial fulfillment of the requirements for the degree of master of science, Department of Veterinary Pathology, Texas A. and M. College, College Station.

Dr. Demke was graduate assistant, Department of Veterinary Parasitology, Texas A. and M. College, while preparing his thesis. He is now located in Dallas, Texas.

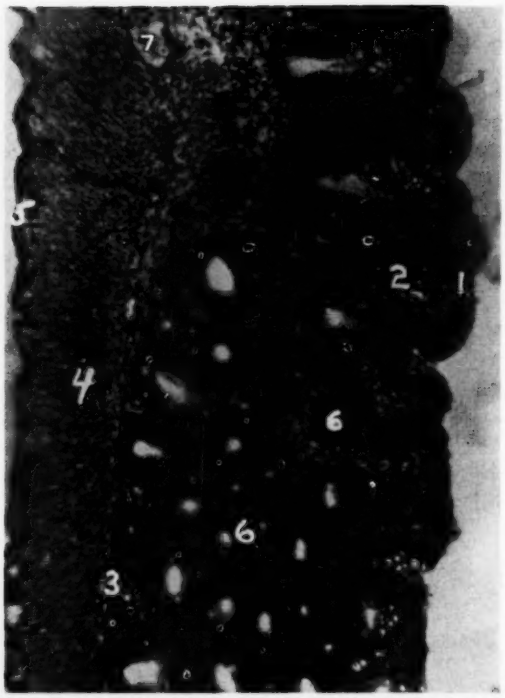


Fig. 2—Section of the cecum of a turkey poult: (1) epithelium, (2) tunica propria, (3) muscularis mucosae, (4) lamina muscularis, (5) serosa, (6) cecal glands, and (7) blood capillary. x 43.3.

fined, continuous, and gave rise to those muscle fibers found in the tunica propria.

The lamina muscularis, heaviest in this portion of the intestine, was well developed and composed of a thick, inner, circular layer and a thin, outer, longitudinal layer with occasional interconnecting fibers at the junction of the two layers.[1, 2, 7]

A few nodules and diffuse areas of lymphoid tissue were found in the duodenum.[1, 7] Small areas, both nodular and diffuse, were also seen in the tunica propria of a poult 24 hours old.

Plexuses of Meissner and Auerbach were present. Fibers from a plexus of Meissner were found in the tunica propria of the villi.

Fibrous connective tissue elements were widely distributed throughout the submucosa, tunica, propria, lamina muscularis, and tunica serosa. Strands were seen extending transversely through the circular layer of the muscularis mucosae.

A few blood capillaries were seen in the serosa and submucosa. Branches from the capillaries in the submucosa extended into the villi. No eosinophilic leukocytes were found in the mucosa or connective tissue stroma in the sections examined.[1, 2, 6, 7]

Small Intestine.—No distinction could be made between the jejunal and ileal portions of the intestine. The villi in both upper and lower parts of the small intestine were somewhat shorter than those found in the duodenum and did not show branching in the sections examined. Their height gradually decreased from the duodenal to the rectal ends.

Simple columnar epithelium with cuticular border covered the villi and was moderately interspersed with goblet cells. Argentaffin cells were found in the epithelium.

The tunica propria was thin but could be followed into the villi and around the glands. Argentaffin cells and lymphoid areas were present to a lesser degree than in the duodenum.

The muscularis mucosae remained well

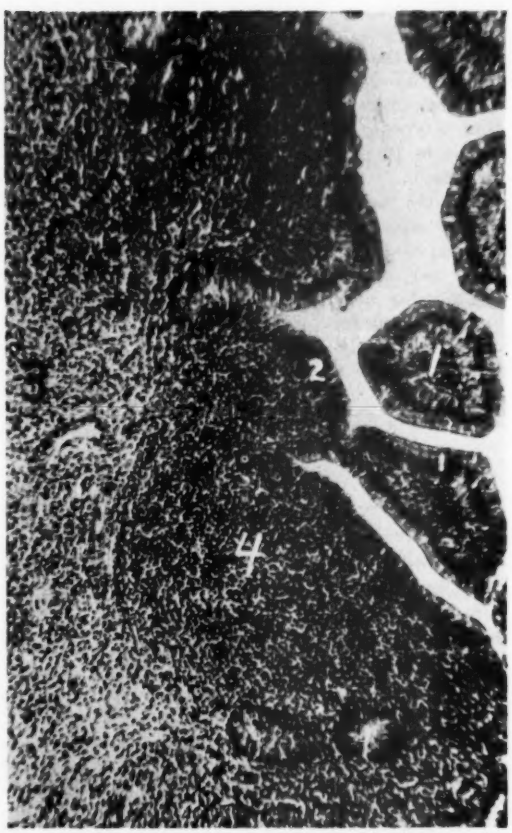


Fig. 3—Section of the rectum of a turkey poult: (1) villus, (2) epithelium, (3) tunica propria, and (4) lymphoid tissue. x 43.3.

defined, continuous, and supplied those fibers in the tunica propria.

The submucosa continued to be poorly developed and lacking in glandular structures.

The lamina muscularis was prominent and was composed of two layers with interconnecting fibers but was slightly thinner than in the duodenum. Fibrous connective tissue elements continued to be prominent.

Lymphoid tissue, blood capillaries, and plexuses of Meissner and Auerbach were found. The lymphoid areas were not as pronounced as in the duodenum and were principally diffuse in nature.

Cecums.—The epithelial cells lining the broad folds and glands of these organs were of a simple columnar type and continued to carry a cuticular border. Many crypts of Lieberkühn were present although no villi were found in the sections examined (fig. 2). The greatest number of goblet cells were in this part of the intestine.[1, 2, 6, 7] Argentaffin cells were numerous in both the epithelium and tunica propria.

The tunica propria was very heavy in this area and contained many argentaffin cells. Numerous nodular and diffuse areas of lymphoid tissue were found. These areas were also found in the cecums of a poult 24 hours old.[2] Fibrous connective tissue elements were present in moderate amounts.

The submucosa was poorly developed, lacked any glandular structures, and contained a few blood capillaries with branches extending into the cecal folds and surrounding the epithelial glands.

The lamina muscularis was well developed, although not as prominent as in the duodenum. Fibrous connective tissue elements were well developed and widely distributed. Many strands extended through the lamina muscularis. A few nerve plexuses were found.

Rectum.—The villi found in the rectum were relatively short and broad. They were covered by simple columnar epithelium with a cuticular border. The epithelium was interspersed with many goblet cells and argentaffin cells. Crypts of Lieberkühn were present, although in less numbers than in the cecums or duodenum. The tunica propria remained thin but contained numerous argentaffin cells.

The muscularis mucosae were well defined while the submucosa was practically nonexistent in the sections examined.

The lamina muscularis continued well defined and divided into two layers. It was not as broad as in other parts of the intestine nor was the outer longitudinal layer as prominent.

Fibrous connective tissue elements were present throughout. Nodules and diffuse areas of lymphoid tissue were only slightly less pronounced than in the cecums (fig. 3). No eosinophilic leukocytes were found. Blood capillaries were decreased in number.

Summary

While the normal histological structure of the small intestine, cecums, and rectum of the turkey poult is essentially similar to that of the chicken, several important differences were noted. The following differences should be kept in mind when pathological conditions are described.

No villi were found in the sections examined from the mid-portion of the cecums.

Argentaffin cells were present in the epithelium and tunica propria from duodenum to rectum, including the cecums.

Lymphoid tissue was present throughout the intestine and cecums and was also seen in the tunica propria of a poult 1 day old.

No eosinophilic leukocytes were found in the mucosa or the connective tissue stroma of the intestine in the sections examined.

References

[1] Bradley, O. C.: The Structure of the Fowl. 3rd ed. J. B. Lippincott Co., Philadelphia (1950): 29-48.

[2] Calhoun, M. L.: The Microscopic Anatomy of the Digestive Tract of *Gallus Domesticus*. Iowa State Coll. J. Sci., 7, (1933): 261-383.

[3] Conn, H. J., and Darrow, M. A.: Staining Procedures. 3rd ed. Biotech Publications, Geneva, N. Y. (1949): (Part I, Section B of loose leaf insert): 18.

[4] Lillie, R. D.: Histopathological Technique. The Blakiston Co., Philadelphia (1948): 101, 228.

[5] Mallory, F. B.: Pathological Technique. W. B. Saunders Co., Philadelphia (1938): 72, 91.

[6] Maximow, A. A., and Bloom, W.: A Textbook of Histology. 5th ed. W. B. Saunders Co., Philadelphia (1949): 399-419.

[7] Trautmann, A., and Fiebiger, J.: Fundamentals of the Histology of Domestic Animals. (Trans. by R. E. Habel and E. L. Biberstein.) Comstock Publishing Co., Inc., Ithaca, N. Y. (1952): 199-216.

The Morphogenesis of the Testes of Chicken Embryos

W. G. VENZKE, D.V.M., Ph.D.

Columbus, Ohio

MOST OF the investigators who have studied the question of the origin of the sex cords and definitive spermatogonia in the male since Waldeyer's [22] article have concluded that the definitive male cells arise either from cells of the germinal epithelium or from primordial germ cells.

LITERATURE CITED

Swift [19] stated that the "primordial germ cells (ureier, keimzellen, gonocytes, ovules mâles, ovules primordiaux, urgeschlechtszellen, and germ cells of the various authors) are found among the cells of the germinal epithelium from its very commencement." These cells were observed in the germinal epithelium of the chick and described by Waldeyer.[22]

Waldeyer,[22] Balfour,[1] and Semon [16] believed that the primordial germ cells arise *in situ* from the epithelial cells in the germinal epithelium by a process of cellular differentiation. Since this work has been done, there has been an increasing belief that the germ cells arise at some region in the embryo at a distance from the future gonad; they then migrate into the region of the wolffian body at approximately the time of the appearance of the germinal epithelium. Nussbaum,[12] Hoffman,[8] Beard,[2] Rubaschkin,[15] Swift,[17] and Goldsmith [6] are of this opinion.

The germ cell crescent of the blastoderm has been removed by Reagan,[14] destroyed with ultraviolet rays by Benoit,[3] destroyed with an electric cautery by Dantschakoff *et al.*,[4] and completely removed by Goldsmith.[7] Histological examination of the blastoderms so treated, and incubated for some time, show the gonadal area free of germ cells.

Willier [24] observed that the gonad, during the indifferent stage of development, is specifically organized with regard to sex. This specific organization is fixed in the four-day embryonic gonads. Willier,[26] using a grafting technique, isolated pieces of tissue from the pellucid area and observed that the gonad which differentiates is invariably sterile, and consists of malelike sex cords; the ovarian cortex never forms.

Willier [23] grew whole blastoderms (about 19 hours of incubation) in chorioallantoic grafts to determine their capacity to differentiate gonads after the crescentic area of the so-called primordial germ cells had been excised. No gonads or other trunk organs developed, and only the anterior organs formed. By transplanting whole blastoderms of later stages (3 and 9 somites), Willier [25] was able to obtain a gonad free of germ cells.

Waldeyer [22] did not believe that germinal epithelium or primordial germ cells had any role in the histogenesis of the male gonad. He was of the opinion that the sex cords originate in the middle of the mesonephros and that they grow into the stroma under the germinal epithelium. He also believed that the primordial germ cells degenerate, and that the mesonephric tissue gives rise to the definitive sex elements.

Before the research of Mihalkowics,[21] the true sex cords were confused with the rete cords with regard to their origin and function. According to Mihalkowics, the rete cords are derived from the mesonephric tubules and the epithelium composing Bowman's capsule. Firket [5] and Swift [18] presented evidence that the rete cords arise as condensations of the mesenchyme between the germinal epithelium and the mesonephros.

Mihalkowics [21] described the true sex cords as arising from the germinal epithelium. He thought that the cells of the germinal epithelium migrate into the subjacent mesenchyme where they group themselves into cords which finally unite with the overlying epithelium. Janosik [9] believed that the sex cords originate as invaginations or processes of the germinal epithelium.

Laulanie [10] expressed the opinion that the primordial gonad is bisexual. He thought that the germinal epithelium is essential in the production of the female elements, and that the male cells are present in the network of cords and develop from the stroma of the gonad. In the germinal epithelium, the cortical ovules are thought to be female while the medullary ovules are male. According to this view, the embryonic gonad is not indifferent but hermaphroditic.

Semon [16] believed that the sex cords are outgrowths of the capsules of the mesonephros. He described two types of cells in the germinal epithelium: the columnar epithelial cells and the large primordial germ cells. Semon also described the stages through which the columnar cells pass in becoming primordial germ cells. His opinion concerning the primordial germ cells is similar to that of Waldeyer.[22] Semon [16] observed that the sexual cords grow out from the malpighian bodies and reach the germinal epithelium about the sixth day of incubation.

Hoffmann [8] employed approximately a dozen species of birds from which evidence was obtained to show that some, if not all, primordial germ cells do not arise in the modified coelomic epithelium.

According to Firket,[5] the cords of first proliferation give rise to the true sexual cords in the male and to medullary cords in the female. The cords of

Dr. Venzke is professor, Department of Veterinary Medicine, College of Veterinary Medicine, The Ohio State University, Columbus.

second proliferation, or the cortical cords, occur and appear later in the female only. The medullary cords in the female go partly into the formation of the ovarian stroma but in general disappear. The cortical cords, or the cords of second proliferation, give rise to the sexual elements and follicular epithelium in the female, according to Swift.[18]

Hoffmann's[8] work showed that the primordial germ cells migrate into the germinal epithelium and then pass into the sexual cord, which develops from the germinal epithelium. Here they become spermatogonia and, finally mature sex cells. Semon[16] stated that the peritoneal mesoblastic cell is the cell from which the spermatozoa develop.

Differentiation manifests itself first in the germinal epithelium and sexual cords. Lillie[11] has observed that in the males, the germinal epithelium never attains as great a thickness as in the females, the sexual cords are better developed, and the stroma less abundant than in the females. The differentiating characters become decisive between the sixth and eighth days of development.

During the sixth and seventh days of incubation, Swift[19] observed that true sexual cords or seminiferous cords arise from the germinal epithelium. Most of the primordial germ cells within the germinal epithelium are carried into the seminiferous cord. The sexual cords remain attached to the germinal epithelium for a very short time. The male sex can easily be recognized at the end of the seventh day of incubation by the nearly equal size of the gonads, the thin germinal epithelium following sexual cord formation, and the presence of few primordial germ cells in the germinal epithelium. During the eighth and ninth days of development, the sexual cords make up most of the volume of the testes. The stroma begins to increase in the eleven-day testes. Prior to the thirteenth day of development, the primordial germ cells in the sexual cords do not divide; at this time, and for the next four days, active division occurs. The primordial germ cells give rise to spermatogonia. Swift further stated that the interstitial cells appear on the thirteenth day and reach their greatest development during the seventeenth day. The interstitial cells are differentiated stroma cells. Lumens appear in the seminiferous cords during the twentieth day of development. The coelomic cells of the germinal epithelium develop into the supporting cells of the seminiferous tubule.

MATERIALS AND METHODS

All embryos used in this study were of a pure single comb, White Leghorn stock. Throughout the study, the fertile eggs were obtained from the same group of hens.

A series of 250 embryos, ranging from twelve hours of incubation to hatching, was used for the morphological studies.

The testes in the developing embryos were fixed in Bouin's fluid or Helly's fluid. All tissues were embedded in Altmann's paraffin mixture and sectioned serially at 6 to 8 μ. Several stains were employed to show the various structures. Hematoxylin-eosin was used as a routine stain. Iron hematoxylin was used to display the cytological structure in those tissues fixed with Helly's fluid. Mallory's connective tissue stain was employed after Helly's fluid to show the connective tissue elements of the testes. The measurements of the testes were made while they were intact within the embryo.

RESULTS

The morphogenesis of the indifferent gonad has been reported previously by Venzke.[20] In most instances, after 177 hours the germinal epithelium of the male chick was reduced to a single layer of cuboid cells. In certain areas, the germinal epithelium appeared as a two-cell layer. At these points, the sexual cords were still attached to the germinal epithelium. However, for the most part, all connections with the germinal epithelium and the seminiferous cords were lost as a result of condensation of mesenchyme, just under the germinal epithelium, to form the tunica albuginea.

The male gonads had a very narrow attachment with the mesonephros. This factor seems to orient the seminiferous cords so that they were arranged obliquely toward the attachment of the gonad. The cords were undulating and were separated from each other by a thin layer of stroma.

The seminiferous cords, which are generally two cells in thickness, were made up of two kinds of cells—peritoneal cells from the germinal epithelium and primordial germ cells. The peritoneal cells were many times more numerous than the primordial germ cells. On longitudinal section of the male gonads, it seemed that more primordial germ cells were found at the anterior and posterior poles than in the central re-

TABLE 1—Average Length by Width Measurements of the Testes of Chicken Embryos with Increasing Age

No. embryos	Age (hours)	Left testis (mm.)	Right testis (mm.)
5	191	2.80 by 0.90	2.40 by 0.60
5	213	3.00 by 0.90	2.50 by 0.70
10	240	3.30 by 1.00	3.00 by 0.80
6	260	3.40 by 1.10	3.10 by 0.80
5	284	3.50 by 1.20	3.20 by 0.90
5	307	4.00 by 1.20	3.50 by 1.10
5	331	4.25 by 1.20	3.70 by 1.10
5	357	5.10 by 1.30	4.00 by 1.20
5	388	6.00 by 1.40	5.00 by 1.20
5	412	6.20 by 1.40	5.50 by 1.20
5	438	6.20 by 1.50	5.70 by 1.25
5	462	6.25 by 1.70	6.00 by 1.30
5	474	6.30 by 1.75	6.00 by 1.25
10	498	6.35 by 1.80	6.10 by 1.50

gion. These cells were small, measuring about 10 μ in diameter. The nuclei were 6 μ in diameter and possessed a clear cytoplasm. For growth changes in the male see table 1.

Histologically, at 191 hours the male testes were little changed except that the tunica albuginea had become more distinct. No mitotic activity was observed among the germ cells. The stroma remained unchanged.

After 206 hours, mitosis was infrequent in the primordial germ cells. Some mitoses were located near the attachment of the gonad, and also near a rich blood supply. The seminiferous cords were more loosely arranged and the stroma had increased slightly.

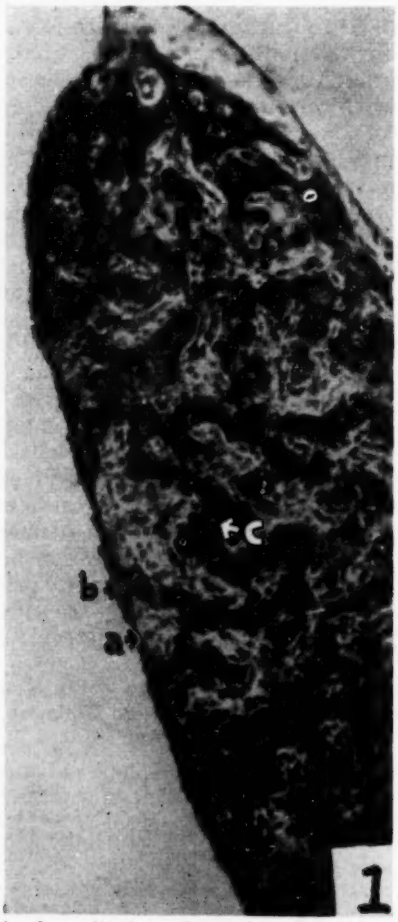


Fig. 1—A sagittal section taken from the testis of a 213-hour-old chicken embryo. (a) Germinal epithelium; (b) primordial germ cell in the germinal epithelium; (c) primordial germ cell in the seminiferous cord. Hematoxylin-eosin stain. x 200.

After 213 hours, a marked change had occurred in the testes (fig. 1). They showed a greatly increased amount of stroma between the seminiferous cords, and the tunica albuginea was thickened. Any increase in size of the testes was largely the result of the increased amounts of connective tissue. The stroma cells had round to oval nuclei measuring 3 to 4 μ in diameter. The cytoplasm was drawn out into processes which anastomosed with neighboring cells.

The seminiferous cords now formed an irregular network and were convoluted. Some cords were only visible in a field for a short distance. The cords were more compact at the anterior and posterior poles than in the central region of the testes.

A definite basement membrane surrounded the seminiferous cords. This membrane was acquired when the cords proliferated from the germinal epithelium. The seminiferous cords were two cells in thickness and are composed principally of the peritoneal-type cell. Rather frequently, a primordial germ cell was found in the seminiferous cords. These two kinds of cells appeared as a syncytium but were easily differentiated on the basis of their nuclei. The primordial germ cell nuclei were from 6 to 8 μ, while the peritoneal cell nuclei were from 3 to 4 μ in diameter. The germ cell nuclei were clear and possessed one or two nucleoli. The seminiferous cords varied in diameter from 11 to 16 μ, depending upon how they were sectioned. The cords were thicker at those points where germ cells were located. Many capillaries were visible in the stroma.

The testes of the 228-hour embryo resembled those of the 213-hour embryo in structure. Increased amounts of stroma caused the testes to become more rounded. The testes were still attached to the mesonephros by a narrow portion of tissue.

The germinal epithelium stained deeply and the cells were of the low cuboidal type. Occasionally, a primordial sex cell was found in the germinal epithelium. The layer of stroma just beneath the germinal epithelium was thicker.

Three kinds of cells now composed the seminiferous cords. The predominating cell was the peritoneal cell. A few spermatogonia were present in the seminiferous cords, and they were similar to the oogonia

in the 228-hour ovary. The primordial germ cells were from 8 to 10 μ in diameter; their nuclei were from 5 to 7 μ in diameter. The spermatogonia measured from 6 to 8 μ in diameter; their nuclei were from 4 to 5 μ. The primordial germ cells and the spermatogonia had no definite position in the seminiferous cords.

The seminiferous cords, as a group, formed a network. They stained deeply and stood out sharply against the lighter connective tissue background. Mitotic figures were present in the nuclei of the peritoneal cells, the primordial germ cells, and the connective tissue stromatic cells.

At 240 hours, many primordial germ cells were present in the seminiferous cords. A few spermatogonia appeared but were widely scattered. The stroma of the testes was more loosely arranged than previously. There was also a slight increase in the amount of stromatic tissue and, therefore, an increase in size of the testes which were almost separated from the mesonephros.

The seminiferous cords at 252 hours had an average diameter of from 20 to 22 μ. Many primordial germ cells were actively dividing, and spermatogonia were present in the seminiferous cords. The peritoneal cells were not active. There was no definite arrangement of cells in the seminiferous cords. Increase in the diameter of the cords had caused the stroma to become more compact.

At 272 hours, the seminiferous cords had an average diameter of 25 μ (fig. 2). All of the sexual elements were seeking a placement against the basement membrane of the cord. The nuclei of the peritoneal cells were also located basally toward the basement membrane. Occasionally, a seminiferous cord contained a small lumen. The lumens, as yet, were not continuous. A few interstitial cells were found within the connective tissue stroma between the seminiferous cords. Many mitotic figures were present among the germ cells.

After 295 hours of development, the testes were cylindrical and had approximately the same diameter on the left and right sides of the body (table 1). They were still attached to the mesonephros by a a very narrow strip of tissue. The sexual cords were increasing in length and were becoming folded, producing a network-like appearance.

The testes at 307 hours were typically elongated and cylindrical. The sexual cells continued to divide in the seminiferous cords. Some cords had lumens which were not continuous. A few interstitial cells were present in the testicular stroma.

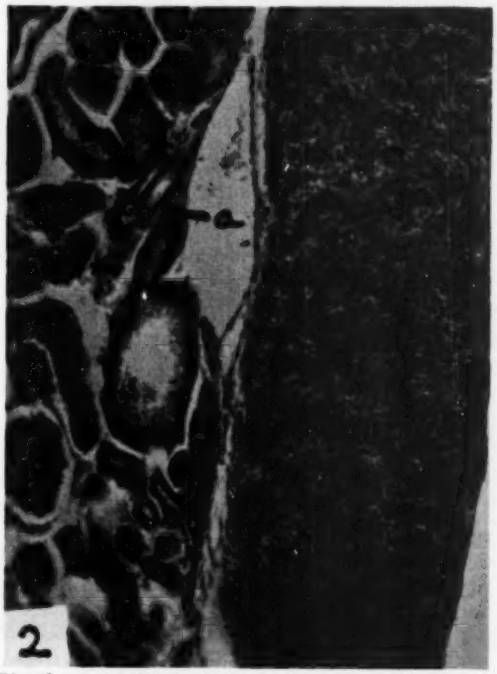


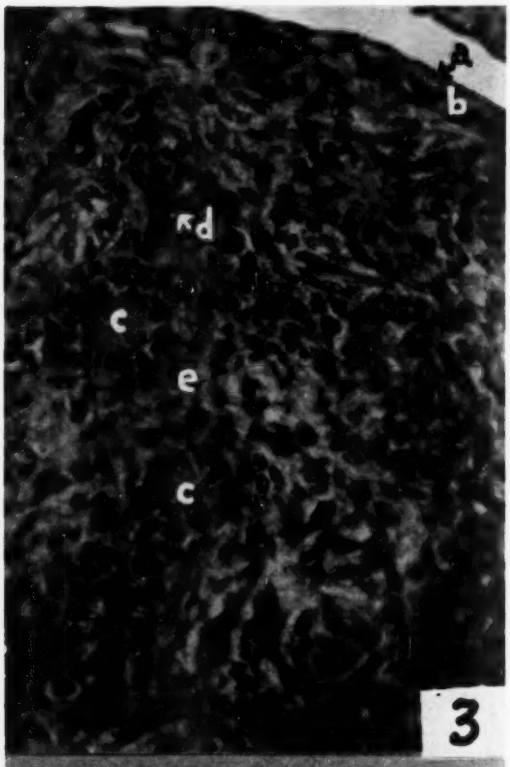
Fig. 2—A sagittal section taken from the testis of 272-hour-old chicken embryo. (a) Mesonephros. Hematoxylin-eosin stain. x 100.

At 331 hours of development, the testes continued to increase in diameter as a result of growth of the seminiferous cords and stroma (fig. 3). The seminiferous tubules varied in diameter from 30 to 35 μ. A slight increase in the number of interstitial cells had occurred, and they were scattered in groups of one to six cells.

The testes at 388 hours remained as previously described except for growth. They seemed to be increasing in length more rapidly than in diameter (table 1).

At 400 hours, the diameter and length of the seminiferous cords had increased. There were many cords which had a diameter between 35 and 40 μ. As a result of growth of the seminiferous cords, there was a crowding of the stroma into strands. Most of the primordial germ cells had divided, giving rise to spermatogonia. Thus far, no evidence had been found regarding maintenance of the peritoneal cells, since no cells

had been found in a phase of mitosis, yet they did not decrease in number. Many of the spermatogonia had assumed a position next to the basement membrane of the seminiferous cord. In many of the cords, lumens were present but were not continuous. There was a very slight increase in the number of interstitial cells.



The testes at 412 hours showed evidence of further increase in size (table 1). Most of the primordial germ cells had changed into spermatogonia by division. Many of the spermatogonia had a peripheral position next to the basement membrane of the cord. A few scattered lumens were present but were not continuous in the seminiferous cords. The interstitial cells had not increased in number. The stroma was becoming very compact.

The testes at 438 hours were elongated and cylindrical in shape and were enveloped by a thin connective tissue layer, the tunica albuginea. The seminiferous cords were composed of two kinds of cells, the peritoneal or supporting cells and the spermatogonia. The supporting cells formed the largest portion of the seminiferous cords. Their nuclei were located side by side near the periphery of the cord, next to the basement membrane. Many of the spermatogonia were located near the basement membrane. The cords seemed to be growing in length and were more branched than previously. There were more interstitial cells present.

After 462 hours, the seminiferous cords were massive, with lumens appearing in many and extending for some distance. The seminiferous cords were growing at the expense of the stroma which was considerably reduced in quantity. The interstitial cells were not increasing.

In the 498-hour embryo, the seminiferous cords had become massive, their diameters varying between 30 and 40 μ (fig. 4). The stroma, in some cases, had been reduced in quantity. The interstitial tissue appeared in amounts similar to that for the 438-hour embryo. The cells within the seminiferous cords had a definite arrangement. The

Fig. 3—A sagittal section taken from the testis of a 331-hour-old chicken embryo. (a) Germinal epithelium; (b) tunica albuginea; (c) seminiferous cord; (d) germ cell in the seminiferous cord; (e) intertubular tissue. Hematoxylin-eosin stain. x 400.

Fig. 4—A sagittal section taken from the testis of a 498-hour-old chicken embryo. Hematoxylin eosin stain. x 200.

nuclei of both the spermatogonia and supporting cells were placed against the basement membrane so that their long axes were at right angles to the long axis of the seminiferous cord. Therefore, the largest amount of cytoplasm in these two kinds of cells was toward the lumen of the cord. Some of the cords had lumens, but these were not continuous throughout the length of the cord.

The cords formed a definite network and extended obliquely toward the remnants of the mesonephros.

DISCUSSION

A discussion of the indifferent gonad prior to 165 hours of development has already been presented.[20] As mentioned in the discussion of the indifferent gonad, it is at 165 hours that morphological sex differences in the gonads become discernible.[20]

The sexual cords, which begin to appear at approximately 134 hours, are the true seminiferous cords. As stated previously, these cords are homologous with the cords of first proliferation in the female.[20] In the male, there is but one series of cords while in the female there are two.

Although the sexual cords begin to appear at 134 hours, it is not until 165 hours that the germinal epithelium reaches its greatest activity in their production. At the end of 165 hours of incubation, the production of the sexual cord ceases. This point is reached at 156 hours, according to Swift.[18]

After 165 hours, it is possible to distinguish between sexes microscopically. The criteria on which this diagnosis is based have been presented.[20] After 191 hours, the sex of the individual can be easily ascertained, either with or without the aid of a microscope.

Seminiferous cords at 177 hours comprised most of the male gonads, since the germinal epithelium had been reduced to a single layer of cuboidal epithelium. The peritoneal cells were many times more numerous than the primordial germ cells in the cords. In the male, as in the left ovary, there were more primordial germ cells at the poles than in the center of the gonad. The primordial germ cells of the male were slightly smaller than those in the left ovary, but they appeared similar in structure.

It was not until 206 hours that the first primordial germ cells were observed in cellular division. There was then a marked difference in the time at which the germ cells in the male began cellular division as compared to those of the female of a similar age. It should be recalled that, during this period of development in the female, the germ cells are in an active mitotic phase and the formation of the cortical cords is beginning to take place.

The amount of stroma between the seminiferous cords had greatly increased at 213 hours (fig. 1). This observation does not agree with that of Swift [19] or Willier,[24] who found that the stroma increased in the testes of the 11-day embryo.

As the testes develop, the seminiferous cords become convoluted. Swift [19] has pointed out that this is the result of adaption by the cords to a space which grows more slowly. On the other hand, this is nature's method of increasing the surface of the seminiferous cords and tubules and maintaining a small compact gland.

After 272 hours, the arrangement of the peritoneal and primordial germ cells was striking. Prior to this time, the primordial germ cells were scattered evenly throughout the cords. After 272 hours, they sought a peripheral placement in the cords (fig. 3). Swift [19] also made this observation but found this change in the placement of the germ cells after the fifteenth day. Semon [16] and Popoff [13] observed that the primordial germ cells have a definite position near the center of the seminiferous cord, while the peritoneal cells occupy the periphery.

It is of interest to note that at 272 hours, the interstitial cells first appeared in the stroma and, as development progressed, these cells became more numerous. Swift [19] records the presence of interstitial cells at thirteen days of incubation. The interstitial cells may be differentiated from the stroma connective tissue cells on the basis of intermediate cell stages among the connective tissue cells. These observations have previously been made by Swift.[19] These interstitial cells varied in shape from round, or cuboidal, to fusiform. The cell was easily differentiated from the ordinary connective tissue cell in that both the nucleus and cytoplasm stained deeply.

Seminiferous cords containing a discontinuous lumen began to appear in about 307 hours. Swift [19] reported cords containing a cavity in the 20-day embryo. Semon [16] observed a lumen in some of the cords in the 17-day embryo. Thus, the time at which the seminiferous cords first contain a lumen is

variable; however, the lumens of the seminiferous cords were not continuous at 498 hours (fig. 4). The seminiferous cords become truly seminiferous tubules sometime after hatching. The lumen results, as Swift [19] has pointed out, by a liquefaction of the cells in the central axis of the seminiferous cord; however, in most cases, it seems that a fissure appears between the cells and proceeds to enlarge.

SUMMARY

The seminiferous cords were discernible at 165 hours of incubation.

Seminiferous cords, after 177 hours of incubation, comprised most of the male gonads because the germinal epithelium had been reduced to a single layer of cuboidal epithelium.

The primordial germ cells were first observed in cellular division at 206 hours.

The intertubular tissue began to increase at 213 hours and continued to increase until hatching.

Interstitial cells differentiated from the intertubular stroma and first appeared at 272 hours.

From 307 hours until hatching, discontinuous lumens appeared in the seminiferous cords.

References

[1] Balfour, F. M.: On the Structure and Development of the Vertebrate Ovary. Quart. J. Micros. Sci., *18*, (1878): 383-438.

[2] Beard, J.: The Germ Cells, Part I. J. Anat. and Physiol., *38*, (1904): 82-105.

[3] Benoit, J.: Destruction des gonocytes primaires dans le blastoderme du poulet par les rayons ultraviolets, aux premiers stades du développement embryonnaire. Proc. 2nd. Internat. Cong. for Sex Res., London, Edinburgh, Oliver and Boyd, (1930): 162-170.

[4] Dantschakoff, W., Dantschakoff, W., Jr., and Bereskina, L.: Keimzelle und Gonade. IA. Identität der Urkeimzellen und der entodermalen Wanderzellen. Experimentelle Beweise. Ztschr. f. Biol., *14*, (1931): 323-375.

[5] Firket, J.: Recherches sur l'organogenise des glandes sexuelles des oiseaux. Anat. Anz., *46*, (1914):413-425.

[6] Goldsmith, J. B.: The History of the Germ Cells in the Domestic Fowl. J. Morphol., *46*, (1928): 275-315.

[7] Goldsmith, J. B.: The Primordial Germ Cells of the Chick. I. The Effect on the Gonad of Complete and Partial Removal of the "Germinal Crescent" and of Removal of Other Parts of the Blastodisc. J. Morphol., *58*, (1935): 537-553.

[8] Hoffmann, C. K.: Étude sur le développement de l'appareil uro-génital des oiseaux. Verhandel. d. k. Akad. v. Wetensch., *1*, (1893): 1-54.

[9] Janosik, J.: Histologisch-embryologische Untersuchungen über das Urogenital-System. Sitzungsb. d. k. Akad. d. Wissensch. Math.-naturw. Cl., Abt. 3, *91*, (1885): 97-199.

[10] Laulanie, M.: Sur le mode d'évolution et la valeur de l'épithelium germinatif dans le testicule embryonnaire du poulet. Compt. rend. Soc. de biol., *3*, (1886): 87-89.

[11] Lillie, F. R.: The Development of the Chick. 2nd ed. Henry Holt and Co., New York, 1919.

[12] Nussbaum, M.: Zur Differenzierung des Geschlechts im Thierreich. Arch f. Mikros. Anat., *18*, (1880): 1-121.

[13] Popoff, N.: L'ovule mâle et le tissu interstitiel du testicule chez les animaux et chez l'homme. Arch. d. biol., *24*, (1909): 433-500.

[14] Reagan, F. P.: Early Castration of the Vertebrate Embryo. Anat. Rec., *11*, (1916): 489-490.

[15] Rubaschkin, W.: Ueber das erste Auftreten und Migration der Keimzellen bei Vogelembryonen. Anat. Hefte., *35*, (1907): 241-261.

[16] Semon, R.: Die indifferente Anlage der Keimdrüsen beim Hühnchen und ihre Differenzierung zum Hoden. Jena. Ztschr. Naturwiss., *21*, (1887): 46-86.

[17] Swift, C. H.: Origin and Early History of the Primordial Germ-Cells in the Chick. Am. J. Anat., *15*, (1914): 483-516.

[18] Swift, C. H.: Origin of the Definitive Sex-Cells in the Female Chick and Their Relation to the Primordial Germ-Cells. Am. J. Anat., *18*, (1915): 441-470.

[19] Swift, C. H.: Origin of the Sex-Cords and Definitive Spermatogonia in the Male Chick. Am. J. Anat., *20*, (1916): 375-410.

[20] Venzke, W. G.: The Morphogenesis of the Indifferent Gonad of Chicken Embryos. Am. J. Vet. Res., *15*, (1954): 300-308.

[21] von Mihalkowics, G. V.: Untersuchungen über die Entwicklung des Harn—und Geschlechtsapparates der Amnioten. Internat. Monatschr. f. Anat. u. Physiol., *2*, (1885): 284-385, 435-485.

[22] Waldeyer, W.: Eierstock und Ei. Ein Beitrag zur Anatomie und Entwicklungsgeschichte der Sexualorgane. Engelmann, Leipzig, 1870.

[23] Willier, B. H.: The Development of Implanted Chick Embryos Following the Removal of the Primordial Germ Cells. Anat. Rec., *34*, (1926): 158.

[24] Willier, B. H:. The Specificity of Sex, of Organization, and Differentiation of Embryonic Chick Gonads as Shown by Grafting Experiments. J. Exptl. Zool., *46*, (1927): 409-465.

[25] Willier, B. H.: The Embryological Foundations of Sex in Vertebrates. (In E. Allen, ed., Sex and Internal Secretions, Chapter IV.) Williams and Wilkins Co., Baltimore, 1932.

[26] Willier, B. H.: Experimentally Produced Sterile Gonads and the Problem of the Origin of Germ Cells in the Chick Embryo. Anat. Rec., *70*, (1937): 89-112.

The Effects of Dietary Deficiencies of Vitamin E and the B Complex Vitamins on the Electrocardiogram of Chickens

PAUL D. STURKIE, Ph.D.; E. P. SINGSEN, Ph.D.; L. D. MATTERSON, Ph.D.; ANNA KOZEFF, B.S.; E. L. JUNGHERR, Ph.D., D.V.M.

New Brunswick, New Jersey and Storrs, Connecticut

VITAMIN E deficiency produces abnormal electrocardiograms (ECG's) in cattle,[1] sheep,[2] rabbits,[3, 4] and monkeys,[5] and histopathological changes in smooth, skeletal, or cardiac muscle of these species respectively.[1, 16, 3, 4, 17]

The changes in the ECG's of E-deficient cattle include prolonged atrioventricular (A.V.) conduction, decreased amplitude of QRS,* right axis deviation and, in some instances, premature systoles (Gullickson and Caverley [1]). The principal changes in the ECG's of E-deficient lambs were prolonged A.V. conduction and changes in amplitude and form of the T wave (Bacigalupo *et al.*[2]).

In chronically E-deficient rabbits, Gatz and Houchin [3] also reported instances of right axis deviation and decreased amplitude of all waves but no change in P-R intervals. In more extensive studies on rabbits, Bragdon and Levine [4] reported a high incidence of heart abnormalities in the latter stages of E depletion. The principal changes in the ECG's were elevated S-T segments and inverted T waves. Chronic E deficiency in monkeys produces a decrease in amplitude of the R and T waves but it produces no changes in the P-R interval or electrical axis (Filer *et al.*[5]).

Diets deficient in E and high in fat produced encephalomalacia in chicks (Pappenheimer and Goettsch [6]), muscular dystrophy in ducks (Pappenheimer and Goettsch [7]), and myopathy of the gizzard in turkeys (Jungherr and Pappenheimer [8]).

The addition of only 1.0 per cent fish oil to a low fat, low vitamin E ration will produce encephalomalacia in chicks (Singsen *et al.*[9]). Increasing the oil level to 2.0 per cent greatly increased the incidence of encephalomalacia. Although chronic vitamin E deficiency does not produce gross lesions in the hearts of chickens, extensive histological studies have not been made. Sections made on the hearts of a few E-deficient birds used in this study revealed nothing abnormal (Jungherr [10]).

This report represents the first on the effects of vitamin E deficiency on the ECG of the chicken.

PROCEDURE

Electrocardiograms were taken at the Storrs Agricultural Experiment Station on adult birds used in an experiment to determine the role of maternal nutrition on the production of encephalomalacia in chicks.

Six groups of Barred Plymouth Rock pullets (1952 series) were housed and fed different diets for a period of 275 days, as shown in table 1. The basal vitamin E-low ration which is shown in table 2 contained 1.69 per cent total fat and 2.54 mg. of total tocopherols per 100 Gm. of ration. This ration (1) was supplemented with adequate amounts of niacin and riboflavin (table 2), but it was deficient in other B complex vitamins (listed in table 1), vitamin K, and ascorbic acid. Fat in the feed was determined by the method as outlined by the Association of Official Agricultural Chemists.[18] Vitamin E was determined in the feed by the method of Swick and Baumann [19] (with feed substituted for animal material), and in blood serum by the method of Quaise *et al.*[20] The blood tocopherol level of birds fed the unsupplemented basal ration dropped to near zero in approximately seven weeks. Diets 1, 3, and 5 were deficient in vitamin E, and 1 and 3 were also deficient in the B complex vitamins listed in table 1. Adequate amounts of vitamin E (*alpha* tocopherol) were added to diets 2, 4, and 6, but diets 2 and 4 were still deficient in these B vitamins. Diet 6 con-

From the journal series, New Jersey Agricultural Experiment Station, Department of Poultry Husbandry, Rutgers University, New Brunswick, N. J.

This work was done at the Storrs Agricultural Experiment Station, University of Connecticut, Storrs, and was supported in part by a grant from the encephalomalacia committee of the American Feed Manufacturer's Association.

Professor and research specialist in poultry physiology, Laboratory of Avian Physiology, Rutgers University, New Brunswick, N. J. (Sturkie); professor and head of department (Singsen), associate professor (Matterson), and assistant instructor (Kozeff), Department of Poultry Husbandry, Storrs Agricultural Experiment Station, University of Connecticut, Storrs; and professor and head of the Department of Animal Diseases, Storrs Agricultural Experiment Station, University of Connecticut (Jungherr).

* The waves of an electrocardiogram have been arbitrarily designated as P, Q, R, S, and T—the order in which they occur during each heart beat.

TABLE 1—Variable Rations Fed to Breeding Hens*

Diet variables	Diet lot No.					
	1	2	3	4	5	6
Basal ration (%)	100.0	100.0	98.0	98.0	100.0	100.0
Vitamin A and D oil (%)**	.....	.....	2.0	2.0	.....	.....
Alpha tocopherol acetate (mg./lb.)	.....	13.62	.....	13.62	.....	13.62
Ca pantothenate (mg./lb.)	.....	.....	.....	.....	5.0	5.0
Pyridoxine (mg./lb.)	.....	.....	.....	.....	2.27	2.27
p-aminobenzoic acid (mg./lb.)	.....	.....	.....	.....	2.27	2.27
Inositol (mg./lb.)	.....	.....	.....	.....	2.27	2.27
Thiamine (mg./lb.)	.....	.....	.....	.....	0.9	0.9
Folic acid (crude 3 %) (mg./lb.)	.....	.....	.....	.....	6.05	6.05
Vitamin K (mg./lb.)	.....	.....	.....	.....	0.045	0.045
Choline chloride (Gm./lb.)	.....	.....	.....	.....	0.3	0.3
Ascorbic acid (Gm./lb.)	.....	.....	.....	.....	0.075	0.075

* Diets of Singsen *et al.*[9] ** 1,500 A and 400 D, I.U. per gram.

tained adequate amounts of E and certain B complex vitamins. Two per cent of vitamin A and D oil (table 2) were added to diets 3 and 4 to study the effects of the oil (fat) on the incidence of encephalomalacia.

This study was concerned not only with the effects of vitamin E deficiency alone on the ECG but also deficiencies of E and B complex vitamins and the B vitamins alone. Electrocardiograms were run on these birds at the termination of the experiment (275 days) with a direct writing electrocardiograph (Carditron), according to the technique of Sturkie.[11] At the close of the 1952 experiments, the surviving old hens from the different lots were placed on a commercial ration for six weeks and then they received the basal ration (1) for approximately six months (1,* table 4, 1953). ECG's were taken on 27 of these hens.

A group of pullets was also fed diet 1, beginning in October, 1952, for eight months when ECG's were taken (1A, table 4). Another group of pullets was fed the basal diet (1) for approximately seven and a half months, and then the diet was supplemented with B complex and other vitamins (table 1), which these birds received for about three weeks before ECG's were taken. This diet is the same as that fed to birds of lot 5 in 1952 (table 4). Electrocardiograms were taken with the Twin-Beam Sanborn electrocardiograph. This is a photographic recording instrument which has a frequency response of 500 cycles per second. ECG's were run on 20 of the chicks hatched from the eggs of vitamin E-deficient hens. These chicks were also fed the basal E-deficient ration for one day to four weeks before ECG's were run, but all of the records were normal.

RESULTS

Effects of the Deficiencies on Reproduction and Mortality.—Singsen *et al.*[9] have reported in detail on this phase of the work for the 1951 to 1952 series. Their results reveal that the basal ration (1), although deficient in E and B vitamins, when fed for approximately nine months did not affect egg production adversely but it decreased fertility significantly.

The mortality for diet 1 during the same period was 45.8 per cent as compared with 12.0 per cent for the normal ration (table 3). Diet 3, however, which was also deficient in E and B vitamins but which contained 2.0 per cent A and D oil, produced a much lower mortality (20.0 %). The mortality for birds receiving diet 1 in 1952 to 1953 was also much lower than for 1951 to 1952 (table 4). Thus, it appears that the E deficiency had no consistent effect on mortality. Diets 2 and 4, which were deficient in B complex vitamins but which were supplemented with vitamin E, also produced high mortality (36.0 %). The birds which received diet 5, which lacked vitamin E but was supplemented with B complex vitamins, experienced a significantly lower mortality (18.9 and 20.0 %). Thus, the high mortality resulting from diets 2 and 4 may be attributed mainly to a deficiency of the B complex vitamins.

Effects of the Deficiencies on the Electrocardiogram.—The percentage of abnormal ECG's and the description of them are shown in tables 3 and 4. Most of these have been previously described by Sturkie[12,13] and were produced by potassium deficiency in the ration. Some of these abnormalities were also produced by acute thiamine deficiency in young chicks (Sturkie[14]). The distribution of the abnormalities, presented herein, was somewhat different from that of the studies mentioned above.

Deficiencies of E and B Vitamins.—Diets 1, 1A, and 3 were deficient in both E and B vitamins. Three different groups received diet 1 (1, 1*, 1A) in two different years. The percentages of abnormal ECG's produced were 22.2, 14.8, and 25.0, respec-

tively (tables 3 and 4). Premature ventricular systoles represented the most common abnormality observed in birds of these groups. These varied from an occasional extra systolic beat to premature beats ex-

TABLE 2—Composition of Vitamin E-Deficient Basal Ration for Pullets* and Amounts of Vitamin Supplements

	(%)
White corn meal**	79.25
Soybean oil meal (50 %)	5.0
Crude casein	7.0
Brewers dried yeast	2.0
Fish meal (60 %)	....
Dicalcium phosphate	3.0
Ground limestone	3.0
Salt	0.5
$MnSO_4$	0.0125
Vitamin A and D feeding oil†	0.2
VITAMIN SUPPLEMENT PER 100 LB. OF BASAL RATION	
Vitamin A (5,000 I.U./Gm.)	70.0 Gm.
Vitamin D_3 (1,500 I.U./Gm.)	25.0 Gm.
Vitamin B_{12} Concentrate (6 mg./lb.)	45.4 Gm.
Niacin	900 mg.
Riboflavin	200 mg.

* Singsen *et al.*[9] **A de-germed, de-branned product manufactured by the Quaker Oats Co., Chicago. † 1,500 A and 400 D I.U. per gram.

hibiting definite rhythms, such as bigeminal and trigeminal rhythms (fig. 1).

Atrioventricular block was observed in only one of the three groups. One bird of this group exhibited second degree A.V. block associated with prolonged A.V. conduction, a condition not hitherto reported in the chicken (fig. 2). Second degree and complete A.V. block represent the most common types of disorders produced by potassium deficiency in chickens.

The mean electrical axes (RS) for the birds of two of these groups were normal, —81.0 and —84.0 degrees. The figure for group 1A, however, was —52.5 degrees and it represents a significant decrease from the normal axis. Twenty per cent of the birds had ECG's in which the R wave in leads II and III was more prominent than the S wave in II and III. This means that the electrical axes of these birds ranged from 0.0 degrees to plus degrees, or it represents right axis deviation. Electrical axes for most normal chicken hearts range from about —70.0 to —110.0 degrees. This means that the S wave in II and III is more prominent than the R wave in these leads but a few normal chickens may show a plus axis (Sturkie [11]).

Diet 3 differed from diet 1 in that it contained 2.0 per cent of A and D oil. Singsen *et al.*[9] have shown that the incidence of encephalomalacia in chicks fed a vitamin E-deficient diet can be increased by increasing the amount of fat or oil in the diet which apparently interferes with the utilization of E or increases the requirement of it. The addition of the oil to the basal diet (1) of adult birds, however, did not increase the incidence of heart abnormalities (table 3). The mean electrical axis for such birds (—56.0 degrees) was significantly lower than normal and of the same magnitude as that reported for group 1A which received no added oil to the diet.

Deficiency of Vitamin E Alone.—Diet 5 differed from diet 1 in that it was adequately supplemented with a number of B vitamins, vitamin K, and ascorbic acid (table 1). Two groups of birds in different

TABLE 3—Mortality* and Description of Electrocardiograms of Hens Receiving Diets Varying in Amounts of Vitamins E, B Complex Vitamins, and Fat (Added Oil) in 1951 to 1952

Lots or diets	1	2	3	4	5	6
Mortality for 275 days (%)	45.8	36.0	20.0	36.0	20.0	12.0
No. of ECG's**	9	16	16	15	20	21
Abnormal (%)	22.2	37.8	26.3	20.0	25.0	4.8
Mean electrical axis (degrees)	—81.0	—72.0	—56.0	—78.0	—86.0	—88.0
Mean heart rate	324	351	331	349	334	324
DESCRIPTION AND PERCENTAGE OF ABNORMAL ELECTROCARDIOGRAMS†						
Premature systoles	22.2	12.5	5.2	6.7	.....	.....
Sinus arrhythmia	6.1	12.5	5.2	6.7	5.0	.....
Bradycardia	.....	.....	5.2	.....	5.0	.....
A.V. block‡	.....	.....	.....	.....	5.0	.....
Elevated S-T	6.1	12.5	.....	6.7	15.0	.....
Low amplitude	.....	.....	.....	.....	20.0	.....
R more prominent than S in II and III, O to plus electrical axis	.....	12.5	21.0	6.7	.....	4.8

* Mortality figures supplied by Singsen *et al.*[9] ** Electrocardiograms. † The percentages exceed 100 because some birds exhibited more than one abnormality. ‡ Atrioventricular.

years received this diet. The incidence of heart disorders was 25.0 per cent for the first group (table 3) and 36.3 for the second (table 4). The latter figure was based on a smaller number of birds (11). The

TABLE 4—Mortality and Description of Electrocardiograms of Hens Receiving Diets Deficient in Vitamin E Only (Lot 5) and Deficient in Both B Complex and E Vitamins (Lots 1* and 1A) in 1953

Lots or diets	1*	1A	5	Control
Mortality (%)	27.5	28.3	18.9	.....
Days on deficiency**	222	271	271	.....
No. of ECG's†	27	20	11	5
Abnormal (%)	14.8	25.0	36.3	0.0
Mean electrical axis (degrees)	−84.0	−52.5	−38.2	−70.0
Mean heart rate	345	334	344	353
DESCRIPTION AND PERCENTAGE OF ABNORMAL ELECTROCARDIOGRAMS				
Premature systoles	3.7	5.0	9.1	
Bradycardia	7.4	5.0	.....	
A. V. block‡	7.4	.....	.....	
R more prominent than S in II and III, O to plus electrical axis	.....	20.0	27.3	

* Old hens, which had been used also in the 1952 experiments. ** Electrocardiograms taken about sixty days before termination of experiment. † Electrocardiograms. ‡ Atrioventricular.

second group received the B vitamin supplement for about three weeks, in contrast to the first group whose diet contained these vitamins from the beginning of the experiment. This may have accounted, in part, for the difference between these groups. Likewise, the types and distribution of the anomalies varied considerably between these groups. The principal disorders exhibited by the ECG's of the 1951 to 1952 group were very low amplitude in some or all of the complexes, and elevated S-T segments. None of these was observed in the 1953 group of birds. The principal abnormality observed in the latter group was a decreased mean electrical axis (−38.2) or right axis deviation. Twenty-seven per cent of the birds had ECG's with more prominent R waves in II and III than S waves. The mean axes for all of the 1951 to 1952 group of birds were normal.

Deficiency of B Vitamins Alone.—Diets 2 and 4 contained adequate amounts of vitamin E but their content of the B vitamins was low, being unsupplemented and containing only the B complex vitamins present in the natural feed stuffs used (table 2). Among the B vitamins, a deficiency of thiamine is probably the most important one in producing heart abnormalities. Sturkie [14] has shown that acute deficiencies of niacin or riboflavin do not produce heart abnormalities but acute thiamine deficiency does. The thiamine content of rations 2 and 4 was approximately 2.63 mg./lb. and, at this level, egg production, fertility, and body weight were not adversely affected (Singsen *et al.*[9]). Obviously, these birds were not completely depleted of the B vitamins. Diet 4 differed from diet 2 in that it was supplemented with 2.0 per cent oil. The percentage of mortality resulting from either diet was the same but the incidence of heart abnormalities was considerably higher for those receiving diet 2 (no oil added). The reason for this discrepancy is unknown.

The total percentage of abnormal ECG's for birds on diets 2 and 4 is of the same magnitude as for those birds on diets deficient in E and B vitamins or E alone. The types and distribution of anomalies of the E-deficient birds were likewise similar to those of the other deficient groups, except that those deprived of B vitamins alone exhibited a considerably lower incidence of right axis deviation than some of the E-deficient groups. The mean electrical axes of

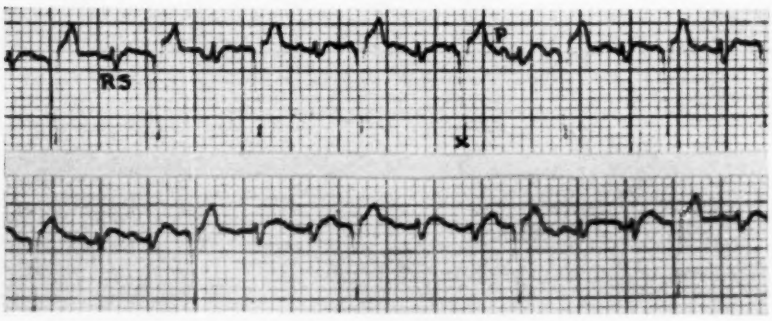


Fig. 1—Premature ventricular systoles exhibiting bigeminal (upper) and trigeminal (lower) rhythm in the electrocardiogram of a chicken. The pauses following the premature beats (indicated by X) are fully compensated. Upper tracing lead II; lower lead III. Chart speed is 25 mm. per second.

Fig. 2—An example of partial atrioventicular (A.V.) block and prolonged A.V. conduction in the electrocardiogram of a chicken. Every fifth ventricular beat (R wave) is blocked. The P-R interval following the dropped beat is shortened, and the interval increases on most successive beats (Wenckebach's phenomenon). The prolonged P-R intervals range from 0.095 immediately following the blocked beat to 0.11 seconds preceding the dropped beat. The heart rate is approximately 370 per minute. Lead I chart speed is 75 mm. per second.

birds on diets 2 and 4 were —72.0 and —78.0 degrees, respectively, within the range for normal birds. However, 19 per cent of the birds (table 3) did exhibit right axis deviation. This condition, then, appears to be rarely associated with thiamine deficiency in birds and in other species, according to the reports of a number of investigators (*see* review by Lepeschkin [15] and report by Sturkie [14]).

DISCUSSION

These results show that deficiencies in the diet of vitamin E alone, E and B complex vitamins, or B complex vitamins alone produce abnormal ECG's in laying hens. The total number of abnormalities and distribution of them varied between different deficiency regimes and even among those receiving duplicate or similar deficiency diets. In some cases, however, the duplicate diets were fed in different years, and this may have accounted for some of the discrepancy.

Diets deficient in E and B complex vitamins (1 and 3), but varying in the amount of oil added, produced variable effects on the ECG's, particularly concerning the types of abnormalities. There was a high incidence of right axis deviation on diet 3 (oil added) but none on diet 1. This difference might be attributed to the added fish oil but, since similar differences were observed among groups of birds fed the same diets (1, 1,* 1A, tables 3 and 4) with no added oil, this conclusion is not justified.

Conditions of the experiment which may have contributed to the variability in results obtained are the number of ECG's taken and the stage of depletion of the birds. In most instances, only one ECG was taken per bird seven to nine months after the initiation of the experiment. Moreover, most of the hens were in apparent good health when the records were obtained. Serial records taken from the beginning of the study and particularly on birds in the terminal stages of depletion, just before death, may have revealed more consistent and reliable findings.

One of the principal anomalies of the ECG's of vitamin E-depleted chickens, not characteristic of B complex-deficient ones, is right axis deviation. This, as indicated in the introduction, has been reported also in other species. Premature ventricular systoles were the most common disorder of vitamin E-depleted birds.

SUMMARY

Adult Barred Plymouth Rock females were fed diets deficient in E and certain B complex vitamins, vitamin E alone, and B complex vitamins alone for approximately seven to nine months.

Vitamin E deficiency alone did not affect egg production or mortality appreciably but it did produce abnormal electrocardiograms (ECG's) in a considerable number of birds.

When the diets were deficient in E and B complex vitamins, the mortality was slightly higher in some groups but egg production was not affected.

The incidence of heart disorders for birds depleted of E and B complex vitamins was no greater than for those depleted of B or E vitamins alone, but the type of anomalies differed somewhat. Right axis deviation was rare in the birds fed the diet deficient in the B vitamins alone, but it was common in the E-depleted, and E- and B-depleted groups. The principal abnormalities of E-deficient hens included right axis deviation, premature ventricular systoles, sinus arrhythmia, and elevated S-T segments. Sinus arrhythmia, *per se*, is not considered abnormal in some mam-

mals and in the pigeon, but it occurs rarely in normal chickens.

References

[1] Gullickson, T. W., and Caverley, C. E.: Cardiac Failure in Cattle on Vitamin E-Free Rations as Revealed by Electrocardiograms. Science, *104*, (1946): 312-313.

[2] Bacigalupo, F. A., Alfredson, B. V., Luecke, R. W., and Thorp, F., Jr.: Electrocardiographic Changes in Vitamin E-Deficient Lambs. Am. J. Vet. Res., *14*, (1953): 214-218.

[3] Gatz, A. J., and Houchin, O. B.: Studies on the Heart of Vitamin E-Deficient Rabbits. Anat. Rec., *110*, (1951): 249-266.

[4] Bragdon, J. H., and Levine, H. D.: Myocarditis in Vitamin E-Deficient Rabbits. Am. J. Path., *25*, (1949): 265-271.

[5] Filer, L. J., Jr., Rumery, R. E., Yu, P. N. G., and Mason, K. E.: Studies on Vitamin E Deficiency in the Monkey. Ann. New York Acad. Sci., *52*, (1949): 284-291.

[6] Pappenheimer, A. M., and Goettsch, M.: A Cerebellar Disorder in Chicks, Apparently of Nutritional Origin. J. Exptl. Med., *53*, (1931): 11-26.

[7] Pappenheimer, A. M., and Goettsch, M.: Nutritional Myopathy in Ducklings. J. Exptl. Med., *59*, (1934): 35-42.

[8] Jungherr, E. L., and Pappenheimer, A. M.: Nutritional Myopathy of the Gizzard in Turkeys. Proc. Soc. Exptl. Biol. and Med., *37*, (1937): 520-526.

[9] Singsen, E. P., Matterson, L. D., Kozeff, Anna, and Bunnell, R. H.: Studies on Encephalomalacia in the Chick. I. The Influence of a Vitamin E Deficiency on the Performance of Breeding Hens and Their Chicks. Poult. Sci., *24*, (1954): 192-200.

[10] Jungherr, E. L.: Unpublished data, 1953.

[11] Sturkie, P. D.: The Electrocardiogram of the Chicken. Am. J. Vet. Res., *10*, (1949): 168-175.

[12] Sturkie, P. D.: Abnormal Electrocardiograms of Chickens Produced by Potassium Deficiency and Effects of Certain Drugs on the Abnormalities. Am. J. Physiol., *162*, (1950): 538-544.

[13] Sturkie, P. D.: Further Studies of Potassium Deficiency on the Electrocardiograms of Chickens. Poult. Sci., *31*, (1952): 648-650.

[14] Sturkie, P. D.: The Effects of Acute Thiamine Deficiency upon the Electrocardiogram of the Chick. Poult. Sci., In press, 1954.

[15] Lepeschkin, E.: Modern Electrocardiography. Vol. 1. The Williams and Wilkins Co., Baltimore, 1951.

[16] Culik, R., Bacigalupo, F. A., Thorp, F., Jr., Luecke, R. W., and Nelson, R. H.: Vitamin E Deficiency in the Lamb. J. Anim. Sci., *10*, (1951): 1006-1016.

[17] Mason, K. E., and Telford, I. R.: Some Manifestations of Vitamin E Deficiency in the Monkey. Arch. Path., *43*, (1947): 363-373.

[18] Official Methods of Analysis of the Association of Official Agricultural Chemists. 7th ed. Official Agric. Chemists, Washington, D. C., 1950.

[19] Swick, R. W., and Baumann, C. A.: Chemical Assay for Tocopherol in Animal Materials. Anal. Chem., *24*, (1952): 758-760.

[20] Quaise, M. L., Scrimshaw, N. S., and Lowry, O. H.: A Micromethod for Assays of Total Tocopherols in Blood Serum. J. Biol. Chem., *180*, (1949): 1229-1235.

A Preliminary Report of Efficiency of Various Mediums for Isolation of Pleuropneumonia-Like Organisms from Exudates of Birds with Chronic Respiratory Disease

H. E. ADLER, D.V.M.; R. YAMAMOTO, B.S.; R. A. BANKOWSKI, D.V.M., Ph.D.

Davis, California

THE PRESENCE of pleuropneumonia-like organisms (PPLO) in exudates of chickens and turkeys affected with chronic respiratory disease (CRD) has been demonstrated in many poultry laboratories throughout the country.[3, 4, 5, 9] Although these organisms have been isolated, cultural methods have not been uniformly successful. It is the purpose of this paper to compare some of the mediums generally employed for the isolation of PPLO with a medium which has been used successfully during the past eighteen months.

MATERIALS AND METHODS

Mediums.—Dehydrated commercially available mediums were used throughout this experiment. All solid mediums were prepared from bacto-PPLO agar (Difco). The enrichment broths were prepared in the laboratory from Difco dehydrated medium according to the formula of Morton *et al.*[6] (This medium did not contain crystal violet.) The only difference between the solid and liquid mediums employed in this comparative study was that the latter did not contain agar. These basic mediums were supplemented with either 10, 20, or 30 per cent horse serum or with 1, 2, or 3 per cent bacto-PPLO serum fraction (Difco). The supplements were added aseptically immediately before use to the sterile broth or agar mediums. The broth was dispensed in 2- or 3-ml. quantities in Kahn test tubes. Penicillin and thallium acetate were incorporated separately or in combination into some of the above mediums as selective bacteriostatic agents. Penicillin was added to the mediums in a concentration of 1,000 units per milliliter after autoclaving. Thallium acetate[2] was added before autoclaving to attain concentrations of 1:2,000 or 1:4,000.

The medium (RM) which was found to be most efficient for the isolation of PPLO was a blood agar slope overlaid with broth (fig. 1). The slope was prepared from bacto blood agar base (Difco) containing 10 per cent fresh defibrinated horse blood and was dispensed in 2-ml. amounts in 1.5 by 12.5 cm. screw-cap tubes. To this agar slope was added the enrichment broth which contained either 20 per cent horse serum or 2 per cent serum fraction with penicillin and thallium acetate in the concentrations specified above.

Inoculums.—To more closely simulate field conditions, it was believed to be more desirable to use exudate from infected birds rather than laboratory-adapted cultures for this study. Four strains of PPLO isolated from field cases of CRD were employed. Each strain was passed through several embryo generations by yolk sac inoculation and then each infected yolk was inoculated into the infraorbital sinus of 3 poults. One additional strain was acquired by inoculating into the infraorbital sinus of 3 poults a suspension of ground trachea prepared from a field case of CRD. The exudates taken from the subsequent sinusitis induced in the poults constituted the source material for the comparison of the cultural methods.

Technique.—Exudates were diluted 1:10 in PPLO broth and 0.05 ml. were inoculated into the enrichment broths and RM mediums. At the same time, the diluted exudate was seeded to the various agars with a 5-mm. loop. After three days' incubation at 37.5 C., the agar plates were exam-

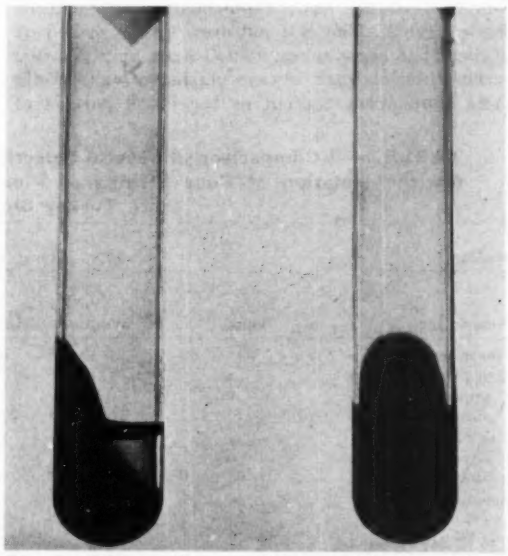


Fig. 1—RM medium, which consists of a blood agar slope and 20 per cent horse serum or 2 per cent serum fraction PPLO broth.

From the Department of Poultry Pathology, School of Veterinary Medicine, University of California, Davis.

This work was supported in part by a grant from Lederle Laboratories, American Cyanamid Company, Pearl River, N. Y.

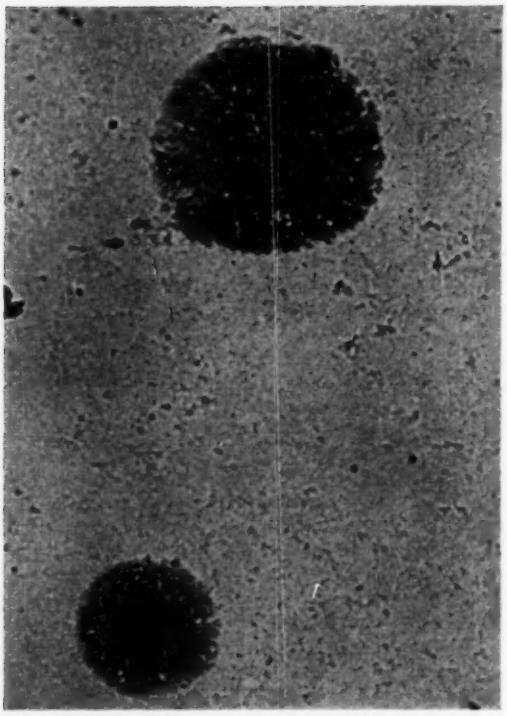


Fig. 2—Photomicrograph of two different types of PPLO colonies stained by the method of Dienes. x 300.

ined for the presence of PPLO colonies, and the enrichment mediums were seeded to agar mediums composed of the same supplements and bacteriostatic agents. The RM cultures were streaked on 20 per cent horse serum PPLO agar or 2 per cent serum fraction agar, always plating to agar of the same supplement content as the broth portion of the medium. If the liquid portion of the RM medium appeared clear, subcultures were made to agar devoid of bacteriostatic agents; however, if the bacterial contaminants appeared to be heavy, as determined by turbidity of the broth portion, subcultures were made to agar plates containing thallium acetate in a concentration of 1:4,000. Previous experience in this laboratory, which was also described by Sabin,[7] has shown that a number of broth-to-broth passages (blind passages) were necessary before some strains of PPLO would grow on agar. With this observation in mind, each inoculated enrichment broth which was plated to solid medium was also serially carried through tubes of enrichment broth composed of the same constituents for at least three blind passages before a sample was discarded. Once a sample yielded positive cultures, the blind passages through enrichment broths were discontinued. The same procedure was followed with the RM medium.

The presence of PPLO was detected by growth on the respective agars as determined by observation with a dissecting scope at 36 diameters, followed by examination of agar blocks stained according to the method of Dienes*[1] at 500 to 1,000 diameters (fig. 2).

RESULTS

As shown in table 1, the largest number of PPLO isolations was obtained using the reference medium (RM). Direct culture of exudates to agar devoid of bacteriostatic agents did not result in a large number of contaminant colonies, but inoculation of exudates into noninhibitory enrichment broths followed by subculture to like agar

* Dienes' stain—methylene blue, 2.5 Gm.; azur II, 1.25 Gm.; maltose, 10.0 Gm.; sodium carbonate, 0.25 Gm.; distilled water, 100 ml.

TABLE 1—A Comparison of Several Selective Horse Serum and Serum Fraction Mediums for the Isolation of Four Strains of Pleuropneumonia-Like Organisms (PPLO) from Turkey Sinus Exudates

Medium	Basal PPLO mediums												Blood slope
Restriction	None			Penicillin-thallium acetate			Penicillin			Thallium acetate			Penicillin-thallium acetate
Horse serum (%)	10	20	30	10	20	30	10	20	30	10	20	30	20
1 to 7 days after appearance of sinusitis	1/D*	3/1	1/1	2/1	2/1	2/1	2/1	2/1	3/1	1/1	1/1	1/1	4/1
7 to 17 days after appearance of sinusitis	0	0	0	0	0	0	0	0	1/3	0	0	0	3/2 to 3
Serum fraction (%)	1	2	3	1	2	3	1	2	3	1	2	3	2
7 to 17 days after appearance of sinusitis	1/1	1/1	1/D	1/3	1/3	1/2	2/3	1/3	1/2	1/3	1/D	1/1	4/1 to 3

* Numerator—number of PPLO strains isolated out of the four strains employed; denominator—number of blind passages in broth required for isolation of the organism; D—direct culture of exudate to agar.

was almost always accompanied by excessive contamination. The addition of 1,000 units of penicillin per milliliter readily controlled the gram-positive contaminants. The combination of thallium acetate 1:4,000 and penicillin appeared to control the growth of *Escherichia coli*.

One subculture from enrichment mediums, including the RM medium, ordinarily sufficed to recover PPLO from the exudates early in the course of sinusitis but, as the disease progressed, three blind passages were required to isolate the organism.

Later in the course of the experiment when samples of exudate were taken seven to seventeen days after appearance of sinusitis, a Proteus organism was encountered. Thallium acetate in a concentration of at least 1:2,000, in addition to the penicillin, was required to control this contaminant in the enrichment and RM mediums. It was still possible to isolate PPLO from the exudates by the RM-enrichment method forty-eight days after sinusitis was first seen.

At this time, several diagnostic laboratories submitted intact frozen chicken tracheas from 20 separate suspected cases of CRD for PPLO culture. Because of the success of the RM medium, this procedure was used exclusively. Fifteen of the 20 cases of suspected chronic respiratory disease were positive for PPLO. Twenty normal chicks raised in isolation were immediately submitted to culture as normal controls. All of these birds were negative after three blind passages on RM medium.

SUMMARY

A comparative study was made for the isolation of pleuropneumonia-like organisms (PPLO) from chickens and turkey exudates. One medium designated as the RM medium, which was composed of a 10 per cent blood agar slope overlaid with a 20 per cent horse serum PPLO broth, appeared to be more satisfactory for the isolation of PPLO than several mediums tested.

References

[1] Dienes, L.: Personal communication.

[2] Edward, D. G.: A Selective Medium for Pleuropneumonia-Like Organisms. J. Gen. Microbiol., *1*, (1947): 238-243.

[3] Grumbles, L. C., Phillips, E., Boney, W. S., and Delaplane, J. P.: Cultural Characteristics of the Agent Causing Infectious Sinusitis of Turkeys and Chronic Respiratory Disease of Chickens. Southwest. Vet., *6*, (1953): 168.

[4] Jungherr, E. L., and Luginbuhl, R. E.: Air Sac Infection in Poultry. Proc. U. S. Livestock San. A., 1952.

[5] Markham, F. S., and Wong, S. C.: Pleuropneumonia-Like Organisms in the Etiology of Turkey Sinusitis and Chronic Respiratory Disease of Chickens. Poult. Sci., *31*, (1952): 902-904.

[6] Morton, H. E., Smith, P. F., and Leberman, P. R.: The Cultivation of Pleuropneumonia-Like Organisms from the Human Genito-Urinary Tract with Reference to Their Possible Venereal Transmission. Am. J. Syph. Gonor. and Ven. Dis., *35*, (1951): 14-17.

[7] Sabin, A. G.: The Filterable Microorganisms of the Pleuropneumonia Group. Bact. Rev., *5*, (1941): 1-68.

[8] Smith, P. F., and Morton, H. E.: The Separation and Characterization of the Growth Factor in Serum and Ascitic Fluid Which Is Required by Certain Pleuropneumonia-Like Organisms. J. Bact., *61*, (1951): 395-405.

[9] Van Roekel, H. W., and Olesiuk, O. M.: Chronic Respiratory Disease of Chickens. Proceedings Book, AVMA, 1952.

Studies of Ntaya, Ilhéus, and Bunyamwera Viruses in Young Dogs

REGINALD L. REAGAN, Major (Ret.), U.S.A.; EDWARD C. DELAHA, B.S.; MILDRED T. STEWART, B.S.; ARTHUR L. BRUECKNER, V.M.D.

College Park, Maryland

THE NTAYA virus (16th mouse passage) used for this study was obtained from Dr. K. C. Smithburn of the Rockefeller Foundation Laboratories in New York. This virus was isolated from mice after intracerebral inoculation of a ground suspension of wild mosquitoes. Its name is applied in respect to the locality where the mosquitoes were caught, the Ntaya swamp in Bwamba County, Uganda Protectorate, British East Africa. Mice develop an encephalitis after intracerebral inoculation (Smithburn and Haddow [1]).

The Ilhéus virus (26th mouse passage) and the Bunyamwera virus (37th mouse passage) were also obtained from Dr. Smithburn. The Ilhéus virus was isolated by Dr. Laemmert in Brazil from mosquitoes (Laemmert and Hughes [2]) and the Bunyamwera virus was isolated in Uganda, Africa (Smithburn, Haddow, and Mahaffy [3]). Both viruses were isolated in mice from mosquito suspensions inoculated into this animal. Mice develop an encephalitis after intracerebral inoculation. This experiment in young dogs was undertaken to determine if they are susceptible to these three viruses.

MATERIALS AND METHODS

Twenty Swiss albino mice (3 weeks old) were inoculated intracerebrally with 0.03 cc. of lyophilized mouse brains (Ntaya virus) of the sixteenth intracerebral mouse passage. The virus received here was lyophilized mouse brain and was diluted to a 10 per cent suspension with sterile distilled water. Twenty Swiss albino mice (3 weeks old) were inoculated intracerebrally with 0.03 cc. of lyophilized mouse brains (Ilhéus virus) of the twenty-sixth intracerebral mouse passage, and 20 Swiss albino mice (3 weeks old) were inoculated intracerebrally with 0.03 cc. of lyophilized mouse brains (Bunyamwera virus) of the thirty-seventh intracerebral mouse passage, using the above technique. Mice from all three groups showed nervous symptoms of mild tremors and convulsions after an incubation period of five to nine days. When symptoms appeared, the mice from each group were destroyed separately and the brain and cord of each were removed aseptically. Pools of Ntaya, Ilhéus, and Bunyamwera mouse brains were ground with alundum and diluted to a 20 per cent suspension with physiological saline. The three suspensions were then centrifuged for five minutes in an angle centrifuge at 2,000 r.p.m. The suspension from each pool was used as the inoculum for initiating the virus experiment in the young dogs. The Ntaya pool was injected intracerebrally into 6 unvaccinated mice and into 6 mice which had been immunized previously with a higher dilution of Ntaya virus over a period of several weeks. After seven days, the unvaccinated mice showed symptoms of central nervous system involvement while the immunized mice showed no nervous symptoms during a 21-day observation period. This confirmed the pool to be Ntaya virus. The Ilhéus and Bunyamwera pools were tested in a similar manner (using Ilhéus- and Bunyamwera-immune mice), and they were confirmed to be Ilhéus and Bunyamwera virus.

Thirty healthy mongrel puppies of 5 to 6 weeks of age and averaging 2 to 3 lb. each were divided

TABLE 1—Response of Young Dogs to Ntaya, Ilhéus, and Bunyamwera Viruses (Mouse-Adapted)

Virus	Route of inoculation	No. of exposed dogs*	Dogs showing symptoms
Ntaya			
	Intracerebral	2	0
	Intranasal	2	0
	Intrarectal	2	0
	Intramuscular	2	0
	Intraperitoneal	2	0
Ilhéus			
	Intracerebral	2	0
	Intranasal	2	0
	Intrarectal	2	0
	Intramuscular	2	0
	Intraperitoneal	2	0
Bunyamwera			
	Intracerebral	2	0
	Intranasal	2	0
	Intrarectal	2	0
	Intramuscular	2	0
	Intraperitoneal	2	0

* These dogs were held for a sixteen-day observation period before being discarded.

From the Virus Department, Live Stock Sanitary Service Laboratory, University of Maryland, College Park. Professor of virus diseases (Reagan); virologist (Delaha); virologist (Stewart); and professor and director of Live Stock Sanitary Service (Brueckner); Virus Department, Live Stock Sanitary Service Laboratory, University of Maryland, College Park.

into 15 groups of 2 puppies each. Both puppies from groups 1 to 5 were each given 1 cc. of a 20 per cent suspension of Ntaya virus by one of the following routes: intracerebral, intranasal, intramuscular, intraperitoneal, and intrarectal. Both puppies of groups 6 to 10 were given 1 cc. of a 20 per cent suspension of Ilhéus virus in the same manner and both puppies of groups 11 to 15 were given 1 cc. of a 20 per cent suspension of Bunyamwera virus in the same manner. Puppies exposed to each virus were kept in separate isolated rooms in the experimental dog kennels. After inoculation, all groups were observed twice daily for nervous symptoms characteristically found in animals infected experimentally with these viruses.

RESULTS

The response of young dogs to the Ntaya, Ilhéus, and Bunyamwera viruses is given in table 1. None of the dogs showed symptoms of central nervous system involvement. They were destroyed after a sixteen-day observation period. The brains of the 15 groups were removed aseptically, ground with alundum to a 20 per cent suspension with physiological saline, and were each inoculated into 6 Swiss albino mice intracerebrally. After a 21-day observation period, the mice showed no symptoms of central nervous system involvement.

DISCUSSION AND SUMMARY

Young dogs were exposed to the mouse-adapted Ntaya, Ilhéus, and Bunyamwera viruses by the intracerebral, intranasal, intrarectal, intramuscular, and intraperitoneal routes. After an observation period of sixteen days, the dogs appeared normal. They were destroyed, the brains were removed aseptically, and a 20 per cent suspension of each was inoculated intracerebrally into mice. The mice appeared normal after a 21-day observation period. Young dogs were not susceptible to these viruses.

References

[1] Smithburn, K. C., and Haddow, A. J.: Ntaya. A Hitherto Unknown Agent Isolated from Mosquitoes Collected in Uganda. Proc. Soc. Exptl. Biol. and Med., *77*, (1951): 130-133.

[2] Laemmert, H. W., and Hughes, T. P.: The Virus of Ilhéus Encephalitis. Isolation, Serological Specificity, and Transmission. J. Immunol., *53*, (1947): 61-67.

[3] Smithburn, K. C., Haddow, A. J., and Mahaffy, A. F.: A Neurotropic Virus Isolated from Aedes Mosquitoes Caught in the Semliki Forest. Am. J. Trop. Med., *26*, (1946): 189-208.

The Detoxification of Chloral Hydrate

LOYAL C. PAYNE, D.V.M., Ph.D.

Ames, Iowa

OF ALL the general anesthetics which have been discovered within recent years, chloral hydrate still remains the anesthetic of choice in large animal surgery. Chloral hydrate has been known as a hypnotic since 1869, although it is extensively used in veterinary medicine for its anesthetic property.

The action of chloral hydrate is certain and prompt, it is lasting, and it is not cumulative. It is cheap, stable, and easily dissolved in water. However, it is very irritating to the tissues and, in certain animals, it is toxic. The prolonged action of chloral hydrate together with this toxicity has caused disfavor among clinicians. Chloral hydrate exists as colorless, transparent crystals, having a pungent odor and a bitter taste. It is believed that chloral hydrate is converted in the body to trichlorethyl-alcohol which is then conjugated principally in the liver with glucuronic acid, forming a nontoxic trichlorethyl-glucuronic acid which is eliminated through the kidneys.

Although certain central nervous system stimulants such as pentylenetetrazol (metrazol), nikethamide (coramine®), and picrotoxin have been administered after chloral hydrate anesthesia in an attempt to overcome the latter's detrimental effects, the results have been unsuccessful. If the physiological detoxification of chloral hydrate in the body could be hastened or assisted in some way, its lasting and toxic effects might be controlled. This suggested that the stimulation of glucuronic acid production in the body might be an aid in controlling the action of chloral hydrate. The following study was undertaken with this purpose in view.

METHODS AND MATERIALS

The method of analytical blood determinations was according to standard procedure. A protein-free filtrate was prepared by the method outlined by Haden.[1] A determination of blood sugar values was made following the standard procedure of Folin and Wu as adapted to the Klett-Sommerson photoelectric colorimeter. The determination of blood chloral hydrate values as outlined by Adams[5] was used in this experiment. Mukerji and Ghose[2] have shown that this method gives the amount of free chloral hydrate in the blood as conjugated chloral hydrate does not enter into this reaction. The determination of the blood alcohol values followed the outline by Nicloux as given by Simmons and Gentzkow.[3]

Known amounts of chloral hydrate (0.1 Gm./lb. of body weight) were administered to the experimental animals. Blood-sugar levels were determined on blood samples drawn before the injection of chloral hydrate. Blood sugar and chloral hydrate levels were determined on blood samples drawn at 15-, 30-, 45-, 60-, and 90-minute intervals subsequent to the injection. By this means, the blood levels of sugar and chloral hydrate in the horses were determined. By these findings, the rate of chloral hydrate disappearance was estimated.

RESULTS AND DISCUSSION

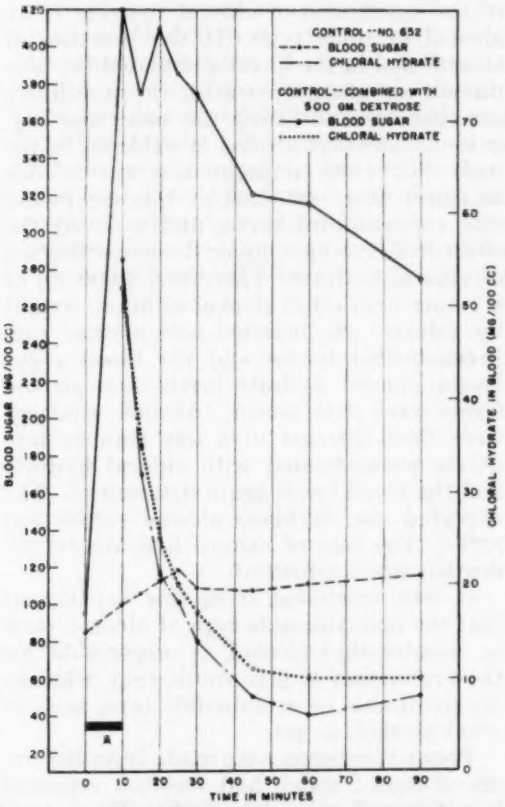
The average values for 18 controlled experiments on 6 different horses are given in table 1. The most significant result seen from these tests was the uniform rise in blood sugar in all cases. The possibility should be pointed out that the glucuronic acid conjugate which is formed after the administration of chloral hydrate may cause an increase in blood sugar levels as it is known that glucuronic acid will reduce alkaline copper reagents. This reduction, however, takes longer than the usual sugar reaction; hence, it may be concluded that the blood sugar rise is a true increase in the blood value. The uniform rate of chloral hydrate disappearance was also noted in the individual animals.

TABLE 1—The Average Blood Sugar and Blood Chloral Hydrate Values in the Blood of 6 Horses in 18 Control Experiments

Post-injection time (minutes)	Blood sugar levels (mg. per 100 cc.)	Blood chloral hydrate levels (mg. per 100 cc.)
0	90	0.0
10	101	40.0
15	102	27.0
30	103	10.5
45	97	8.0
60	104	7.5
90	107	7.0

Dr. Payne is assistant professor, Department of Veterinary Physiology and Pharmacology, Iowa State College of Agriculture and Mechanic Arts, Ames.

Injections of dextrose, previous to the injection of chloral hydrate, subsequent to the injection of chloral hydrate, and combined with the chloral hydrate solutions

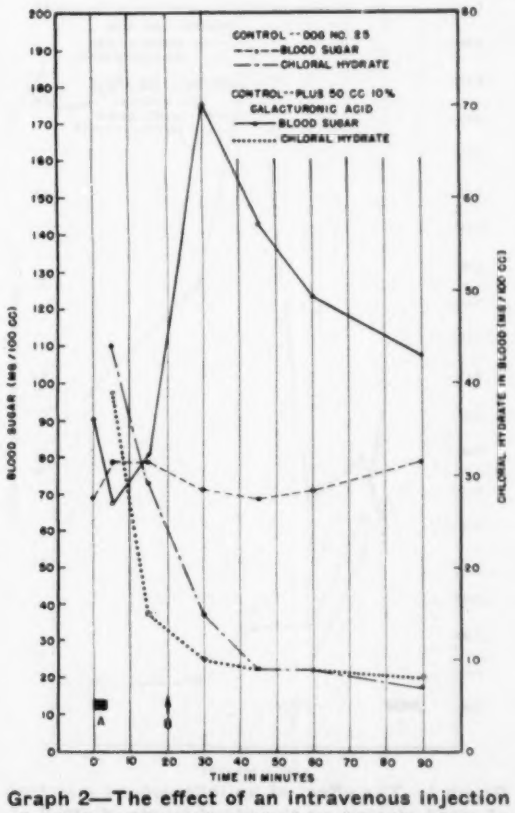


Graph 1—The effect of dextrose combined with chloral hydrate on the blood levels of sugar and chloral hydrate in a horse. (A—period of injection.)

(graph 1), failed to hasten the disappearance of chloral hydrate. These injections were made at different intervals, as follows: one hour previous to chloral hydrate administration, two hours previous, at the time of administration, and one hour subsequent to the administration of chloral hydrate. The injection of dextrose at the same time as the chloral hydrate administration appeared to increase the rate of chloral hydrate disappearance the most.

In like experiments, the injection of insulin (200 units), insulin (80 units) combined with 500 cc. of 5 per cent dextrose, epinephrine (5 cc. of 1:1,000), glycine (50 cc., 100 cc., and 250 cc. of a 10 % solution), lactic acid (1,000 cc. of a 5 % solution), and ethyl alcohol (1,000 cc. of a 10 % solution) failed to alter the detoxification of chloral hydrate in the blood.

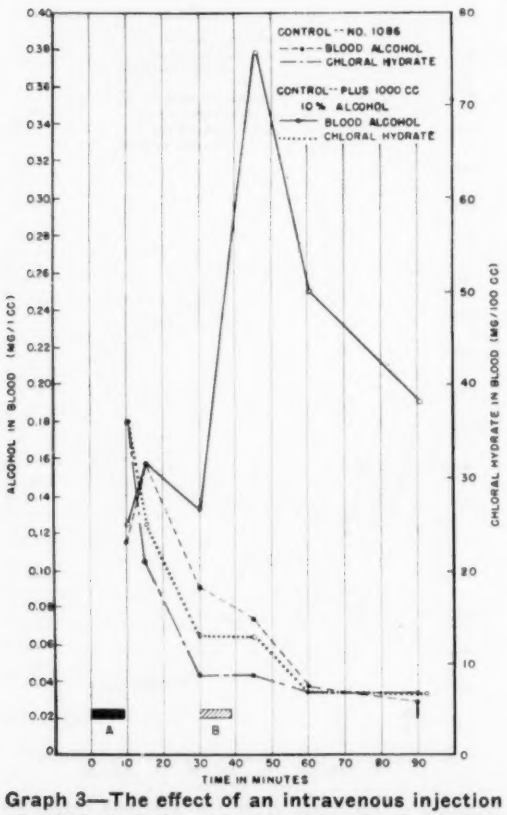
The final experiment conducted in this investigation was the intravenous injection of a solution of galacturonic acid (50 cc. of a 10 % solution) into a dog premedicated with chloral hydrate (graph 2). If the body is capable of conjugating the free form of hexuronic acid, either glucuronic acid or galacturonic acid with toxic principles, a decrease in the blood level of chloral hydrate should result. Because of the limited amount of galacturonic acid available, only one trial was made using this compound. There was no noticeable decrease in the blood values of chloral hydrate in the dog in this one experiment as compared with the control. Everett [4] states



Graph 2—The effect of an intravenous injection of galacturonic acid on the blood levels of sugar and chloral hydrate in a dog premedicated with chloral hydrate. (A—period of chloral hydrate injection, B—period of galacturonic acid injection.)

that the ingested glucuronic acid can not be utilized by the body for conjugation and this may well be true for galacturonic acid injected into the blood stream.

Many investigators studying the mechanism of chloral hydrate hypnosis believe this compound is converted within the body to trichlorethyl alcohol; hence, alcohol levels were determined in several of the experimental animals which had been given chloral hydrate and, in each case, a distinct increase in the blood alcohol level was noted (graph 3). The method of blood alcohol determination was based on the distillation of the alcohol away from the specimen and then determining the amount of alcohol present by the change in the color of the dichromate solution. This method eliminated the possibility of interfering substances such as glucose, lactic acid, or other carbohydrates. An increase in the blood alcohol from 0.0 mg. per cubic centimeter of blood to 0.158 mg. of alcohol per cubic centimeter of blood immediately after the injection of chloral hydrate was indicative of the conversion of chloral hydrate to an alcohol of some type. If the presence of this alcohol in the blood stimulated the production of glucuronic acid, the possibility of other alcohols doing the same was suggested. As ethyl alcohol is oxidized in the body and is not conjugated, it was decided to inject large amounts of this compound into experimental horses and to study the effect it might have upon the detoxification of chloral hydrate. Therefore, 1,000 cc. of a 10 per cent ethyl alcohol solution, weight by volume, was injected into normal nonpremedicated horses and the blood sugar levels, chloral hydrate levels, and alcohol levels were determined. Alcohol solutions were then injected in a like manner into horses premedicated with chloral hydrate and the blood levels again determined. The expected rise in blood alcohol values was noted. The rate of chloral hydrate detoxification was unchanged.



Graph 3—The effect of an intravenous injection of ethyl alcohol on the blood levels of ethyl alcohol and chloral hydrate in a horse premedicated with chloral hydrate. (A—period of chloral hydrate injection, B—period of ethyl alcohol injection.)

It was concluded from this experiment that the nonoxidizable type of alcohol, such as trichlorethyl alcohol, is responsible for the production of glucuronic acid, whereas the oxidizable or combustible type, such as ethyl alcohol, is not.

Tissue specimens were made from the livers of horses which had received repeated injections of chloral hydrate. The examination of these sections showed very little change within the liver tissue. The detrimental effects of other halogenated compounds, such as chloroform and carbon tetrachloride upon the liver is well known. However, repeated chloral hydrate injections into experimental animals apparently caused no liver damage.

The results from this experiment would indicate that the injection of chloral hydrate into the experimental horses caused a definite increase in the blood sugar level of these animals. Also, that the disappearance of chloral hydrate from the blood and its elimination from the body is very rapid immediately after the administration of this compound. The complete elimination of chloral hydrate from the body does not occur until two or three days after the injection, as was shown by the resid-

ual level of chloral hydrate in the blood two and three days after chloral hydrate administration.

SUMMARY

Studies were made on the blood levels of sugar, chloral hydrate, and alcohol after intravenous injections of chloral hydrate solutions into experimental animals (horses). The results of intravenous injections of insulin, insulin and dextrose combined, glycine, ethyl alcohol, and galacturonic acid had little effect upon the disappearance of chloral hydrate from the blood stream. Tissue sections of the livers of 2 of the animals which had received repeated injections of chloral hydrate solutions showed no alteration in the reaction of these organs.

References

[1] Haden, R. L.: A Modification of the Folin-Wu Method for Making Protein-Free Blood Filtrates. J. Biol. Chem., *56*, (1923): 469-471.

[2] Mukerji, B., and Ghose, R.: Urina y Clearance of Free Chloral in Normal and Liver-Damaged Dogs and the Possibility of Using It as a Test for Liver Function. Indian J. M. Res., *27*, (1940): 757-764.

[3] Simmons, J. S., and Gentzkow, C. J.: Laboratory Methods of the United States Army. 5th ed. Lea and Febiger, Philadelphia (1944): 343-345.

[4] Everett, M. R.: Medical Biochemistry. 2nd ed. Paul B. Hoeber, Inc., New York (1946): 332.

[5] Adams, W. L.: The Determination of Chloral Hydrate, Chloroform, and Related Substances in Blood. J. Pharmacol. and Exptl. Therap., *74*, (1942): 11-17.

The Differential Effect of Basic Fuchsin and Thionin on *D*-Alanine Utilization by the Genus Brucella

H. S. CAMERON, D.V.M., Ph.D., and MARGARET E. MEYER, B.S.

Davis, California

SINCE Huddleson [1] demonstrated that *Brucella abortus*, *Brucella suis*, and *Brucella melitensis* could be differentiated by the bacteriostatic action of basic fuchsin and thionin, the fundamental problem concerning the mode of action of these dyes has been under consideration but is still unclarified.

Recent reports reveal that there exist significant metabolic differences between the three species of Brucella. Cameron, Holm, and Meyer [2] demonstrated that *Br. abortus* and *Br. melitensis* metabolized glutamic acid with equivalent oxidative rates, and that one of the mechanisms involved in the utilization of glutamic acid was a urea cycle. *Brucella suis*, however, did not metabolize this amino acid. Altenbern and Housewright [3] reported finding both an active glutamic-alanine and aspartic-glutamic transaminase in *Br. abortus* strain 19. Cameron and Meyer [4] have shown that comparable enzyme systems exist in *Br. melitensis* but they were unable to demonstrate them in *Br. suis*. They also reported that the three species of Brucella differed quantitatively in their oxidative rates on substrates of *d*- and *l*-alanine, and expressed the view that these differences reflected some fundamental variation in the pathways of alanine metabolism. Marr and Wilson [5] demonstrated a *d*-alanine racemase in *Br. abortus*. This may explain why this species oxidizes both alanine isomers at an equal rate. The presence of this enzyme in the other two species has not been demonstrated.

In view of the differences in dye bacteriostasis between the species, and the fact that these reported variations in enzyme activity are involved directly or indirectly in alanine utilization, it seemed appropriate to determine if an interference in alanine metabolism was one of the factors in dye bacteriostasis.

MATERIALS AND METHODS

The resting cell suspensions were prepared as described by Cameron and Meyer.[4] The activity of the resting cells was measured by conventional Warburg techniques (Umbreit *et al.*[6]). The temperature of the water bath was 38.5 C. One milliliter of cell suspension was used in each flask, and the total flask content was 3.0 ml. The dyes and the *d*-alanine were dissolved in Sorenson's buffer and prepared so that the desired concentrations were added to the side arm in 1-ml. quantities, and the vessels were brought to volume with buffer when necessary. In the first phase of the work, the flask contents were equilibrated for twenty minutes, and then the dye was tipped in. In the second part of the study, the *d*-alanine and dye were tipped simultaneously after equilibration. Endogenous rates of oxygen uptake were determined for each experiment and all rates of exogenous respiration were measured, at least in duplicate.

RESULTS

The results are summarized in the following tables:

Table 1 represents the basic response of resting Brucella cells to basic fuchsin and thionin. It will be noted that basic fuchsin and thionin depressed the rate of respiration in *Br. abortus* and *Br. suis*. With *Br. melitensis* the rate was depressed by basic fuchsin only.

TABLE 1—Effect of Basic Fuchsin and Thionin on Resting Cell Respiration of the Three Species of Brucella

Dye concentrate	$QO_2(N)$ Values* with dyes					
	Basic fuchsin			Thionin		
	Br. abortus	*Br. suis*	*Br. melitensis*	*Br. abortus*	*Br. suis*	*Br. melitensis*
250 μg.	−26	−46	−15	− 9	9	13
500 μg.	−34	−47	− 6	− 2	3	24
750 μg.	−27	−53	−12	−22	2	46
1,000 μg.	−35	−69	−36	−12	8	32
1,250 μg.	−36	−68	−39	−10	− 4	28
1,500 μg.	−33	−66	−41	−11	− 9	29
1,750 μg.	−27	−74	−28	− 8	−14	26
2,000 μg.	−34	−68	−51	−25	−24	16
2,250 μg.	−30	−74	−50	−26	−31	18
2,500 μg.	−36	−66	−49	−30	−29	24
2,750 μg.	−27	−70	−49	−22	−22	21
3,000 μg.	−31	−71	−48	−22	−20	25
Endogenous	59	89	76	64	89	76

* $QO_2(N)$ = Cubic millimeters of oxygen used by an organism under standard conditions; endogenous rates subtracted.

From the School of Veterinary Medicine, University of California, Davis.

Table 2 shows the effect of the dyes on the metabolism of *d*-alanine by the three species. It will be observed that basic fuchsin inhibited the oxidation of *d*-alanine by *Br. suis* but not by *Br. abortus* or *Br. melitensis*. Thionin, on the other hand, retarded the oxidation rate by *Br. abortus* but not by *Br. suis* or *Br. melitensis*.

DISCUSSION

The effect of basic fuchsin and thionin on resting *Br. abortus*, *Br. suis*, and *Br. melitensis* was in accord with the effect on the growing cell. Because of the number of factors such as oxidation-reduction reactions, cell permeability, and toxicity of the dyes to oxidative enzymes that may be involved in dye bacteriostasis, the results from this type of an investigation are difficult to interpret. Because of this, it was considered that the data would be more meaningful if presented on two complementary phases of the problem. The first object then was to determine the basic response of Brucella cells to the dyes. Although this information was fundamental to interpreting the second phase of the study, it was not, *per se*, information of a conclusive nature. The aspect of the problem concerning cellular response to the dyes, particularly dyes that can be reversibly oxidized and reduced, has been studied by Barron and Hoffman [7] who concluded that such dyes act as catalysts for the oxidative processes within the cell and stimulate oxygen consumption, provided that the cell membrane is permeable to the dye.

From table 1 it may be seen that the only significant stimulation that occurred was in the case of *Br. melitensis* in response to thionin. All other respiration rates were depressed considerably below the endogenous rate, although thionin and basic fuchsin are both capable of being reversibly oxidized and reduced. Another aspect of this problem was reported by Page and Robinson [8] who, by studying the inhibition by dyes of *Staphylococcus aureus*, determined that bacteriostasis could not be correlated with the actual oxidation-reduction potential at which the dye is reduced.

Although it is not possible to evaluate the role of cell permeability and oxidation-reduction activity from the results in table 1, there are some conclusions that can be suggested by combining the information on both tables. The respiration of *Br. melitensis* was depressed by basic fuchsin and stimulated by thionin; yet in the presence of either dye it oxidized *d*-alanine. Thus, it would appear that factors other than oxidation-reduction are involved in bacteriostasis. Using Barron and Hoffman's [7] criterion of stimulation of oxygen uptake as the basis of interpretation, we may conclude that basic fuchsin does not permeate any of the Brucella species, and that thionin permeates only *Br. melitensis*. Since *Br. abortus* and *Br. melitensis* both oxidized *d*-alanine in the presence of basic fuchsin, it is apparent that, although the basic response was one of depression, the dye was neither lethal to the cell nor was it interfering with the permeability of the cell to *d*-alanine.

This leaves two possible modes of action. One of these is that the cell membrane of *Br. suis* was rendered impermeable to *d*-alanine by basic fuchsin, even though *Br. abortus* and *Br. melitensis* behaved differently. It would appear that if this were the mechanism of bacteriostasis, then *Br. abortus* should also be rendered completely impermeable to *d*-alanine by thionin, which is not the case. At 1,750 μg. of thionin the oxidation rate of *d*-alanine by *Br. abortus* was still one third of the rate without the dye; with 1,750 μg. of basic fuchsin the rate was one half. At higher concentrations of the dyes, this dropped to one fourth and one third, respectively. In the case of *Br.*

TABLE 2—Differential Effect of Basic Fuchsin and Thionin on D-Alanine Utilization by the Three Species of Brucella

	$QO_2(N)$ Values* with 10 mg. *d*-alanine and dyes					
	Basic fuchsin			Thionin		
Dye concentrate	*Br. abortus*	*Br. suis*	*Br. melitensis*	*Br. abortus*	*Br. suis*	*Br. melitensis*
250 μg.	70	18	130	63	162	99
500 μg.	65	7	98	57	128	70
750 μg.	55	−37	110	50	123	93
1,000 μg.	50	−41	112	30	99	91
1,250 μg.	50	−51	92	18	74	111
1,500 μg.	46	−47	120	29	80	106
1,750 μg.	45	−52	119	28	72	94
2,000 μg.	40	−58	10	21	77	104
2,250 μg.	31	−55	10	24	54	102
2,500 μg.	32	−40	−20	22	69	98
2,750 μg.	30	−42	−25	20	55	90
3,000 μg.	32	−56	−21	6	50	86
0	85	156	120	83	156	105

* $QO_2(N)$ = Cubic millimeters of oxygen used by an organism under standard conditions; endogenous rates for *Br. abortus* 45-90; *Br. suis* 82-117; *Br. melitensis* 62-118 are subtracted.

melitensis, the thionin did not interfere significantly with the oxidation rates regardless of concentration. Basic fuchsin from 250 μg. to 1,850 μg. stimulated the oxidation rate and concentrations above that amount caused inhibition. With *Br. suis*, the complete inhibition of *d*-alanine utilization by basic fuchsin leaves the question of cell permeability in doubt. Data, however, from another phase of these investigations, which will be reported in a subsequent paper, showed that lactic acid oxidation by *Br. suis* was not inhibited by this dye, indicating that the cell was not rendered impermeable by basic fuchsin.

The data, therefore, would suggest that one of the mechanisms involved in differential bacteriostasis is the toxicity of basic fuchsin and thionin to different enzyme systems. The data also emphasize the fact, already suggested by Cameron and Meyer,[4] that there is a variation in alanine metabolism within the genus.

SUMMARY

The effect of basic fuchsin and thionin on the oxidation of *d*-alanine by the three species of Brucella was studied. Data were obtained from resting cell respiration studies suggesting that one of the mechanisms responsible for the differential bacteriostatic action of these dyes lay in their effect on different enzyme systems.

References

[1] Huddleson, I. F.: The Differentiation of One Species of the Genus Brucella. Tech. Bull. 100, Agric. Exper. Sta., Michigan State College. 1929.

[2] Cameron, H. S., Holm, L. W., and Meyer, Margaret E.: Comparative Metabolic Studies on the Genus Brucella. I. Evidence of a Urea Cycle from Glutamic Acid Metabolism. J. Bact., *64*, (1952): 709-712.

[3] Altenbern, R. A., and Housewright, R. D.: Alanine Synthesis and Carbohydrate Oxidation by Smooth *Brucella Abortus*. J. Bact., *62*, (1951): 97-105.

[4] Cameron, H. S., and Meyer, M. E.: Comparative Metabolic Studies on the Genus Brucella. II. Metabolism of Amino Acids That Occur in the Urea Cycle. J. Bact. (in press).

[5] Marr, A. G., and Wilson, P. W.: The Alanine Racemase of *Brucella Abortus*. Arch. Biochem. and Biophysics (in press).

[6] Umbreit, W. W., Burris, R. H., and Stauffer, J. F.: Manometric Techniques and Related Methods for the Study of Tissue Metabolism. Burgess Publishing Co., Minneapolis, 1945.

[7] Barron, E. S. G., and Hoffman, L. A.: The Catalytic Effect of Dyes on the Oxygen Consumption of Living Cells. J. Gen. Physiol., *13*, (1930): 483-494.

[8] Page, J. E., and Robinson, A. F.: An Examination of the Relationship Between Bacteriostatic Activity and the Normal Reduction Potentials of Substituted Quinones. Brit. J. Exptl. Path., *24*, (1943): 89.

Assay of Antibiotics by Use of Methylene Blue Milk

I. A. SCHIPPER, D.V.M., and W. E. PETERSEN, Ph.D.

St. Paul, Minnesota

THE DETERMINATION of antibiotic levels in various body fluids and/or secretions has become a common laboratory procedure, the results of which are widely employed in defining the range and relative efficacy of various antibiotics and antibiotic preparations. Various methods of determining levels of antimicrobic agents have been described.[1]

One of the first methods described for the determination of chlortetracycline (aureomycin) concentration was by Dornbush *et al.*[2] This method was utilized by McCulloch *et al.*[3] in determining chlortetracycline concentration in milk following intramammary infusions. In attempting to utilize the McCulloch method of assay of chlortetracycline, difficulties were encountered which have been presented.[4] A modification of the McCulloch method was utilized for preliminary investigation of chlortetracycline[5] and terramycin®[6] levels in milk, blood, and urine. Later investigations definitely indicated that further modifications of the above method of assay were necessary to provide greater accuracy and ease in determining levels of these antibiotics in milk, blood, and urine.

MATERIALS AND METHODS

The medium utilized consists of 10 Gm. of dried nonfat milk solids dissolved in 100 ml. of distilled water with a pH corrected to 7.0. Observations indicated less difficulty in obtaining a satisfactory medium if the pH adjustments were made previous to the addition of the dried nonfat milk solids to the distilled water. The milk solution is autoclaved for ten minutes at 15 lb. of pressure. Additional autoclaving produces a darker colored milk preparation that is less desirable in determining the end-point reading following incubation. Portions of each sample to be assayed are placed in sterile ½ by 4½-in. Wassermann tubes as indicated in table 1.

The methylene blue solution is prepared at four times the concentration prescribed in "Standard Methods for the Examination of Dairy Products." Ten milliliters of this solution is added to each 100 ml. of the dried milk preparation. The increased methylene blue concentration is utilized to compensate for the dilution occurring in each tube and to provide a more definite end-point reading.

The organism utilized is *Bacillus mesentericus*. This organism was selected because of its ability to reduce methylene blue and its sensitivity to chlortetracycline and terramycin as well as its lack of pathogenicity to those working with it. The organism is grown in penicillin assay broth for eighteen hours and diluted 1:100 in the methylene blue milk solution. It is then incubated for fifteen minutes at 37 C. before addition to the Wassermann tubes.

The antibiotic standard is prepared from a frozen stock solution containing 1 mg./ml. This solution is diluted, with sterile distilled water, to 0.5 μg. per milliliter for terramycin and 0.2 μg. per milliliter for chlortetracycline. The standard end-point reading for terramycin is 0.075 to 0.1 μg. per milliliter. The end-point reading for chlortetracycline is 0.05 to 0.06 μg. per milliliter.

When the process of dilution is completed, as shown in table 1, all tubes are stoppered with sterile rubber stoppers and inverted at least once. The tubes are then incubated in a water bath at 37 C. for four hours or until all control samples are completely reduced and the standard sample end point is within the prescribed range. The end point is the greatest dilution of unknown or standard samples at which no reduction of methylene blue occurs. All tubes are inverted once every thirty minutes after the first hour of incubation, with the final inversion made thirty minutes previous to reading.

DISCUSSION

Experimentation with known quantities of antibiotics in milk, blood serum, and urine has definitely indicated the methylene blue milk method of assay to be an efficacious means of determining levels of chlortetracycline and terramycin in these secretions. A similar method of determining the antibiotic concentration in milk, by

TABLE 1—Method for Assay Procedure with Methylene Blue Milk

Tube	Dried milk preparation (ml.)	Sample standard or control (ml.)	Methylene blue milk plus test organism (ml.)	Dilution factor
1	0.0	1.0	1.0	2.00
2	0.1	0.9	1.0	2.22
3	0.2	0.8	1.0	2.50
4	0.3	0.7	1.0	2.857
5	0.4	0.6	1.0	3.33
6	0.5	0.5	1.0	4.00
7	0.6	0.4	1.0	5.00
8	0.7	0.3	1.0	6.66
9	0.8	0.2	1.0	10.0
10	0.9	0.1	1.0	20.0

From the Division of Dairy Husbandry, (Peterson) University Farm, St. Paul. Dr. Schipper is associate professor, Department of Veterinary Science, College of Agriculture, Fargo, N. D.

employing bromcresol purple as an indicator, has been outlined by Edwards and Haskins.[7] These workers experienced difficulty in determining antibiotic concentrations in blood serum with the methylene blue milk method of assay.

Heatley[8] has reviewed the numerous factors that may influence the various tests available to evaluate the antibiotics. It is obvious that the methylene blue milk method of assay is not infallible and that influencing factors, described by Heatley, must always be considered. The suggested employment of this method is based on its comparative accuracy, ease of preparation, and rapidity of obtaining results.

When employing the methylene blue milk method of assay, it must first be determined if any of the secretions or blood serum of each animal contain any inhibitory substances. If any inhibitory substances are present, end-point readings will be obtained in the complete absence of antibiotics. This condition was found to exist in the urine, milk, and blood serum of some animals. This condition was not persistent for any one animal over a greater period than eight days.

Attempts have been made to utilize this method of assay for antibiotics other than chlortetracycline and terramycin; however, thus far, this method has proved unsatisfactory in that difficulty has been encountered in isolating test organisms that will satisfactorily reduce methylene blue milk and possess adequate sensitivity.

SUMMARY

1) A method of determining the quantity of terramycin® and chlortetracycline (aureomycin) in milk, blood serum, and urine has been outlined.

2) The method outlined eliminated the necessity of processing (coagulation and filtration) the milk sample and provided a rapid means of determining antibiotic concentrations, usually with end points easily determined.

References

[1] U. S. Department of Health, Education and Welfare; Food and Drug Administration, Washington, D. C. Compilation of Regulations for Tests and Methods of Assay and Certification of Antibiotic and Antibiotic-Containing Drugs. May 1, 1953.

[2] Dornbush, A. C., and Pelcak, E. J.: The Determination of Aureomycin in Serum and Other Body Fluids. Ann. New York Acad. Sci., *51*, (1948): 218-220.

[3] McCulloch, Ernest C., Kiser, J. S., and Migaki, Hoyo: Aureomycin in the Treatment of Staphylococcic Mastitis in Cows. Vet. Med., *44*, (1949): 253-258.

[4] Schipper, I. A., and Petersen, W. E.: Assay of Aureomycin in Milk by Use of Methylene Blue. Vet. Med., *46*, (1951): 222-224.

[5] Schipper, I. A., and Petersen, W. E.: Milk, Blood, and Urine Concentrations of Aureomycin After Intramammary Infusions and Intravenous Administrations. Vet. Med., *47*, (1952): 367-371.

[6] Schipper, I. A., and Petersen, W. E.: Milk, Blood, and Urine Concentrations of Terramycin Following Intravenous, Intramammary, and Intramuscular Administration. Vet. Med., *48*, (1953): 315-318.

[7] Edwards, S. J., and Haskins, M. D.: The Determination of Antibiotic Levels in Blood and in Milk Following Parenteral and Intramammary Injection. J. Comp. Path., *63*, (1953): 53-67.

[8] Heatley, N. G.: Methods for Measuring the Sensitivity of Microorganisms to Antibiotics. Antibiotics, *1*, (1949): 200-214.

Susceptibility and Serological Response of Various Species of Animals to Infection with the Virus of Vesicular Stomatitis

T. KOWALCZYK, Vet. Dipl., D.V.M., M.S., and C. A. BRANDLY, D.V.M., M.S.

Madison, Wisconsin

THE PURPOSE of these studies was to ascertain whether the susceptibility of various species of domestic and laboratory animals to artificial exposure to vesicular stomatitis virus (VSV) was correlated with the circulating serum titer of specific virus-neutralizing antibodies resulting from such experience with VSV.

The specific virus-neutralizing or protective antibodies can be detected and titrated by injecting mixtures of the VSV and the serum under test into suitable host organisms, e.g., embryonating eggs (Burnet [2]).

Long and Olitsky [13] infected guinea pigs with New Jersey and Indiana type strains of VSV by injecting the skin of the foot pads. Challenge of immunity was accomplished with homotype and heterotype VSV, ten to fourteen days later. It was found that infection by the intramuscular routes protected against pad reinfection with the homotype virus ten days later.

Cox and Fite [5] employed mice for testing the specific neutralization of VSV. Inoculation was intracerebral and the observation period was fourteen days. Burton [3] observed natural reinfection of horses in less than two months after recovery from the malady. Frank *et al.*[8] observed that after recovery from mucosal exposure, cattle and horses were refractory to infection by intracerebral inoculation. Hanson [10] observed in mice a similar refractivity to intracerebral inoculation following recovery from intraperitoneal or intramuscular inoculation of homotypic VSV.

Cotton [4] reported the induction of signs of infection by multiple injections of immune cattle with VSV at short intervals of time. The route, dosage, and intervals of time were not recorded. Development of immunity as a result of inapparent infection by contact with infected animals in the acute stage of the disease was observed in swine by Shahan.[15]

MATERIALS AND METHODS

The virus used for the estimation of the titer of the virus-neutralizing antibodies in the serums of various species was the same as that employed in experiments of infectivity of this virus for animals (Kowalczyk [11]) with the New Jersey-type Wisconsin strain (Brandly *et al.*[1]).

Exposure of the animals to VSV was by several routes. The inoculum, Wisconsin strain virus, consisted of chorioallantoic fluid of infected 11-day-old chicken embryos from the eighth serial passage. The fluid was collected from embryos dead two days after inoculation. Titrated each time before use, the values were 10^4 to 10^5 embryo l.d.$_{50}$.

Serums were collected from experimentally infected animals fourteen days after initial exposure. The animals were exposed immediately after the collection of a pre-injection blood sample. After another fortnight, a second blood sample was taken. Blood was obtained from the vena cava, jugular vein, or by cardiac puncture, depending on the species. General anesthesia was accomplished with nembutal or ether. The blood samples were left at room temperature for twenty-four hours to allow clotting and syneresis. The serum was removed aseptically to separate glass tubes. All samples were inactivated for thirty minutes at 56 C. and cultured aerobically for bacteria. After bacterial sterility was confirmed, the serums were stored at —25 C. for future use.

One horse was injected intracerebrally and in the oral mucosa, another intravenously. Injections were made, intravenously, intracerebrally, and intramucosally in cows, sheep, goats, swine, dogs, ferrets, guinea pigs, and chickens. Rabbits were inoculated intravenously and intramucosally. Separate animals of these species were injected by each route.

For evaluation of response of the different species of animals, a mixture of equal quantities of inactivated serum and respective decimal dilutions of the virus fluid were prepared, shaken, and incubated at 25 C. for thirty minutes. Each mixture was injected into a series of 4, 9-day-old chicken embryos, and the deaths of embryos were recorded during the succeeding four days. The titer of the serum was based on neutralization of the lowest virus dilution to the extent that 50 per cent or more of the inoculated chicken embryos survived. The neutralizing titer value was calculated as the difference between the exponential numbers of the titers of the virus and serum, e.g., if the serum neutralized 10^2 l.d.$_{50}$ units of virus which had a 10^5 titer, its neutralizing value was $5 - 2 = 3$ or arithmetically 1,000 l.d.$_{50}$ per 0.1 ml. of inoculum.

Paper NS No. 132 from the Department of Veterinary Science, University of Wisconsin, Madison. Published with the approval of the director of the Wisconsin Agricultural Experiment Station.

Department of Veterinary Science (Kowalczyk), Departments of Veterinary Science and Bacteriology (Brandly), University of Wisconsin, Madison.

Appreciation is expressed to Dr. R. P. Hanson for valuable suggestions during the course of this study.

RESULTS

Both horses, 1 receiving injections intramucosally and the other intravenously, developed neutralizing titers of 10^3 fourteen days after initial exposure and titers of 10^4 after the second injection.

A heifer exposed intracerebrally showed a titer of 10^2 and 10^3 after the first and second inoculation, respectively. A cow infected via the oral mucosa and reinfected fourteen days later developed titers of 10^4 and 10^5, respectively.

The goats, regardless of whether they were infected via the oral mucosa, intravenously, or intracerebrally, developed identical titers of 10^2 after the first injection and 10^3 and $10^{2.5}$ after the second exposure (fig. 1).

Swine developed similar antibody values after exposure by each route, oral mucosa, intravenous, and intracerebral. The initial and reinjection titers were 10^4 and 10^5, respectively.

The 3, 1-year-old dogs, inoculated in the oral mucosa, intravenously, and intracerebrally, responded identically to the first inoculation with a titer of 10^3. After reinfection, a titer of 10^4 per 0.1 ml. was observed, whereas in the 11-week-old dogs, the initial and reinjection titers by each route were 10^2 and 10^4, respectively.

One of the 4 rats inoculated intracerebrally survived and showed an antibody titer of 10^3 following the second injection. A neutralizing antibody titer of 10^2 was demonstrated in rats after both the first and second exposure by intradermal injection of the foot pads.

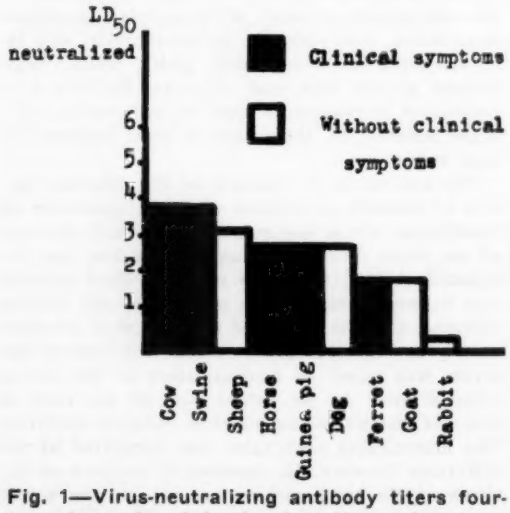


Fig. 1—Virus-neutralizing antibody titers fourteen days after injection into the oral mucosa of various species of animals with vesicular stomatitis virus.

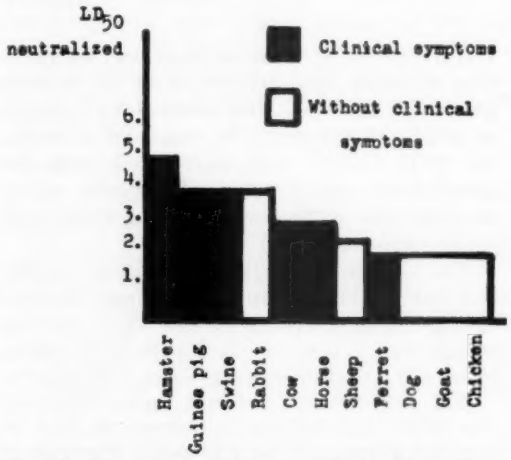


Fig. 2—Virus-neutralizing antibody titers fourteen days after intravenous injection into various species of animals with vesicular stomatitis virus.

After the second intraperitoneal inoculation, 4 hamsters, 4 months old, and 2 hamsters, 5 weeks old, developed titers of 10^4. The second intracardial exposure resulted in a titer of 10^5 by 3 of the 4-month-old hamsters.

Two guinea pigs after intracerebral and intracardial inoculation developed identical antibody titers, namely, 10^4 after the first and 10^5 after the second exposure. Intradermal and oral mucosal inoculation incited 10^3 and 10^4 titers after the first and second exposure, respectively. The lowest antibody response was after corneal scarification. The titers after both injections were the same, 10^2.

Two ferrets, each of which were inoculated intramucosally, intracardially, intracerebrally, intranasally, and by corneal scarification, developed the same titers, *i.e.*, 10^2 after the first and 10^3 after the second exposure.

Two groups of chickens, 6 weeks old and 1 year old, responded similarly with serum titers of $10^{1.5}$ after the first and of 10^3 after the second inoculation. After the first nasal instillation, the titer was $10^{1.5}$ in both 6-week-old and 1-year-old birds.

After the second instillation, the titer was 10^2. Intravenous injection of both groups of chickens resulted in the development of identical titers of 10^2 after the first inoculation; the second VSV injection produced

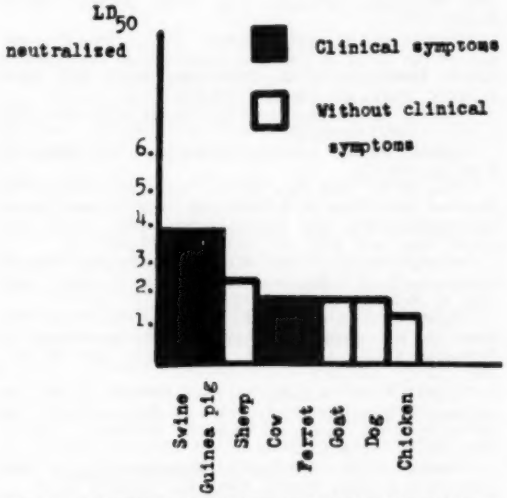


Fig. 3—Virus-neutralizing antibody titers fourteen days after intracerebral injection into various species of animals with vesicular stomatitis virus.

serum titers of $10^{2.5}$ in the 6-week-old birds and of 10^3 in the 1-year-olds.

The 4 rabbits did not respond with antibody formation to inoculation of the oral mucosa. Those receiving 10 ml. of active, egg-propagated virus intravenously developed 10^4 titers of VSV neutralizing antibodies. Ten months later, when the titer of the 1 surviving rabbit had almost disappeared, it was reinoculated with 10 ml. of virus and the titer returned to 10^4 within fourteen days.

Two chinchillas receiving injections intradermally into the foot pads and 2 others inoculated in the oral mucosa developed $10^{2.5}$ antibody titers. Reëxposure by nasal instillation twenty days later raised the titer to 10^3.

Discussion

In general, the degree of acquired specific immunity of an animal is correlated with the titer of virus-neutralizing antibodies in its serum. This correlation usually is greater and more uniform during the time the antibody titer serum is increasing than during the period of antibody decline. A high titer of virus-neutralizing antibodies developed in guinea pigs as a result of intradermal footpad infection, but the other previously uninoculated footpad was still susceptible to infection. Circulating antibodies probably do not penetrate the superficial tissue such as those of the footpad in a quantity which is adequate to protect these tissues against infection.

Fazekas de St. Groth, Donnelley, and Graham [7] described uneven distribution of influenza antibodies in their studies on immunology of this disease. They introduced killed influenza virus into mice by intraperitoneal, subcutaneous, and intranasal routes and obtained the same level of serum antibodies in every individual. They observed, however, significant differences in the concentration of antibodies in the fluid secretions at the surface of the respiratory tract and the antibody level of the serum. After intranasal instillation of the antigen, the concentration of specific antibodies on the surface of the respiratory tract was much higher than that which followed the introduction of the antigen by the intraperitoneal or subcutaneous routes. The authors concluded that specific resistance to influenza does not correspond to the level of the blood serum antibodies but to the concentration of antibodies on the surface of the respiratory tract. By means of a so-called "pathotopic antigen" they were able to shift the antibodies from the serum to the surface of the respiratory tract and to accumulate there a sufficient concentration for protection against respiratory challenge.

Two horses after one month and 2 ferrets after two months were refractive to challenge by the same route, whereas a heifer with a high titer of virus-neutralizing antibody was susceptible to reinfection one month after primary infection.

The three species — the dog, goat, and chicken—which were refractive to natural or artificial infections with VSV responded to intravenous injection with antibody titers (10^2) as high as those produced by the relatively much more susceptible ferrets.

Ferrets and cattle exposed intracerebrally responded with antibody levels equal to those of goats and dogs, 10^2. Titers twice as great, however, developed in swine and guinea pigs.

Direct correlation between the antibody titer response and the native susceptibility

of the several species of animals to reinfection obviously was not demonstrable. Cattle and swine, both naturally susceptible to VSV, developed the highest titers (10^4) of all the species exposed intramucosally. The same route of exposure resulted in titers of 10^3 in horses, guinea pigs, and dogs; the first two species are highly susceptible whereas the third may be considered highly resistant. Ferrets which are very susceptible and goats which are resistant developed titers of 10^2 after intramucosal infection. Only hamsters developed antibody titers of 10^5 after intracardial infection. The rabbit, resistant to all routes of infection, responded with the same level of antibodies as did swine and guinea pigs exposed to large doses of virus by the intravenous route.

SUMMARY

An attempt was made to correlate the degree of immunity of several species of animals with the titers of vesicular stomatitis virus-neutralizing antibodies produced by them in response to different routes of exposure. Likewise, the anamnestic response to subsequent injection after two weeks was ascertained. The titers of neutralizing antibodies ranged from 10^2 to 10^4 or even 10^5 and were not fully correlated with the natural susceptibility of the particular species of animal.

The rabbit proved to be resistant to artificial infection yet produced antibodies of high titer. A titer of 10^4, however, was reached only after a relatively large quantity of virus antigen was injected intravenously. A response, apparently "anamnestic," was observed in all species, and there were no marked differences in the degree of the response among the different species. The anamnestic antibody response therefore did not appear to represent an index of the resistance or susceptibility of a given species.

References

[1] Brandly, C. A., Hanson, R. P., and Chow, T. L.: Vesicular Stomatitis with Particular Reference to the 1949 Wisconsin Epizoötic. Proc. Book, AVMA (1951): 61-67.

[2] Burnet, F. M., and Galloway, I. A.: The Propagation of the Virus of Vesicular Stomatitis in the Chorioallantoic Membranes of the Developing Hen's Egg. Brit. J. Exptl. Path., *15*, (1934): 105-113.

[3] Burton, A. C.: Stomatitis Contagious in Horses. Vet. J., *73*, (1917): 234-242.

[4] Cotton, W. E.: Vesicular Stomatitis. Vet. Med., *22*, (1927): 169-175.

[5] Cox, H R., and Fite, G. L.: Serological Distinction Between the Virus of Encephalitis in St. Louis Equine Encephalomyelitis and Vesicular Stomatitis. Proc. Soc. Exptl. Biol. and Med., *31*, (1934): 499-500.

[6] Fazekas de St. Groth, S.: Studies in Experimental Immunology of Influenza IX. Austral. J. Exptl. Biol. and M. Sci., *24*, (1951): 339-351.

[7] Fazekas de St. Groth, S., Donnelley, M., and Graham, D. M.: Studies in Experimental Immunology of Influenza VIII. Austral. J. Exptl. Biol. and M. Sci., *24*, (1951): 323-337.

[8] Frank, A. H., Appleby, A., and Seibold, H. R.: Experimental Intracerebral Injection of Horses, Cattle, and Sheep with the Virus of Vesicular Stomatitis. Am. J. Vet. Res., *6*, (1948): 28-38.

[9] Findlay, G. M.: Non-Specific Resistance Against Virus Infection. J. Roy. Micros. Soc., *48*, (1948): 20-47.

[10] Hanson, R. P.: Personal communication, 1951.

[11] Kowalczyk, T.: The Response of Domestic and Laboratory Animals to Initial and Secondary Exposure to Vesicular Stomatitis Virus. Master's degree thesis, University of Wisconsin, Madison, 1952.

[12] Kowalczyk, T., and Brandly, C. A.: Experimental Infection of Dogs, Ferrets, Chinchillas, and Hamsters with Vesicular Stomatitis Virus. Am. J. Vet. Res., *54*, (1954): 98-101.

[13] Long, P. H., and Olitsky, P. K.: Immunity in Guinea Pigs to the Virus of Vesicular Stomatitis. Proc. Soc. Exptl. Biol. and Med., *23*, (1928): 478-480.

[14] Topley, W. W. C., and Wilson, G. S.: Principles of Bacteriology and Immunity. Vol. II. Williams and Wilkins Co., Baltimore, (1946): 1225-1244.

[15] Shahan, M. S., Frank, A. H., and Mott, L. O.: Studies of Vesicular Stomatitis with Special Reference to a Virus of Swine Origin. J.A.V.M.A., *108*, (1946): 5-19.

[16] Sorensen, D. K.: Virus Infectivity Transmission and Immunity Studies of Vesicular Stomatitis in Cattle and Sheep. Ph.D. thesis, University of Wisconsin, Madison, 1953.

[17] Wagener, K.: Investigation on the Pathogenicity of Vesicular Stomatitis Virus. Cornell Vet., *21*, (1931): 344-359.

Response of the Green Turtle (*Pseudemys Elegans*) to Several Virus Strains after Intracerebral Inoculation

REGINALD L. REAGAN, Major (Ret.), U.S.A.; EDWARD C. DELAHA, B.S.; MILDRED T. STEWART, B.S.; A. L. BRUECKNER, V.M.D.

College Park, Maryland

THE VIRUSES employed in this study were the Newcastle disease virus strains (California, Colorado, Kentucky, Connecticut, Delaware, and Minnesota, BAI, 1946), Zika and Ntaya viruses,[1] Wyeomyia virus,[2] Bwamba virus,[3] Semliki Forest virus,[4] West Nile virus,[5] Bunyamwera virus,[6] Ilhéus virus,[7] Uganda "S" virus,[8] Anopheles "A" and Anopheles "B" viruses,[8] and a street virus rabies strain accession number 192482. All of the above viruses except the Newcastle disease virus strains (BAI, 1946) and the rabies virus strain were furnished by the American Type Culture Collection in Washington, D. C. (Public health permit was obtained to work with these viruses.) These viruses were obtained by the above institution from Dr. K. C. Smithburn of the Rockefeller Foundation Laboratories in New York, except for the Newcastle disease virus strains which were obtained in 1946 from the Bureau of Animal Industry, U.S.D.A., Washington, D. C., and the rabies virus strain which was obtained from Dr. A. N. Metcalf of the Pennsylvania Bureau of Animal Industry at Enola. This specimen had been proved positive for rabies at the Pennsylvania Bureau of Animal Industry and had been preserved in glycerine before this specimen's trial in turtles.

MATERIALS AND METHODS

Three-week-old Swiss albino mice (Webster strain) and young green turtles (*Pseudemys elegans*) were employed in this study. The mouse strain was the one recommended by Webster[9] as being especially susceptible to neurotropic viruses.

Groups of 6, 3-week-old Swiss albino mice were inoculated intracerebrally with 0.03 cc. of lyophilized mouse brain reconstituted to volume from each virus strain of the 12 to be confirmed. The strain consisted of the thirty-seventh mouse passage of the Bunyamwera strain, the 204th mouse passage of the Wyeomyia strain, the twentieth mouse passage of the Uganda "S" strain, the twenty-sixth mouse passage of the Ilhéus strain, the 165th mouse passage of the Anopheles "A" strain, the 175th mouse passage of the Anopheles "B" strain, the eighty-second mouse passage of the Bwamba strain, the 146th mouse passage of the Zika strain, the sixteenth mouse passage of the Ntaya strain, the sixth mouse passage of the Semliki Forest strain, the twenty-ninth mouse passage of the West Nile strain and of the dog brain of the rabies strain.

After an incubation period of four to eight days, the mice from all groups showed nervous symptoms, mild tremors, and paralysis. When symptoms appeared, the sick mice from each group were killed, and the brains were removed aseptically and kept in separate pools for each strain. Pools of each strain of infected mouse brains were ground with alundum and diluted to a 20 per cent suspension with physiological saline. The 12 infected mouse-brain pools were then centrifuged for five minutes in an angle centrifuge at 2,000 r.p.m. The supernatant fluids were removed from all 12 pools separately and used as inoculums.

Each virus pool except the rabies pool was injected intracerebrally into 4 unvaccinated mice and 4 vaccinated mice. The mice were vaccinated by injection with high dilution virus suspensions of extremely low virulence given over a period of several weeks. In six to ten days the unvaccinated

TABLE 1—Response of the Green Turtle (Pseudemys Elegans) to Intracerebral Inoculation of Neurotropic Viruses

Virus strain	Turtles exposed (No.)	Turtles showing central nervous system involvement (No.)
Bunyamwera	4	0
Bwamba	4	0
Zika	4	0
Ntaya	4	0
Wyeomyia	4	0
Uganda "S"	4	0
Ilhéus	4	0
Anopheles "A"	4	0
Anopheles "B"	4	0
West Nile	4	0
Semliki Forest	4	0
NDV (California)	4	0
NDV (Colorado)	4	0
NDV (Connecticut)	4	0
NDV (Delaware)	4	0
NDV (Kentucky)	4	0
NDV (Minnesota)	4	0
Rabies	4	0

NDV—Newcastle disease virus.

From the Virus Laboratory, Live Stock Sanitary Service, University of Maryland, College Park. Professor of virus diseases (Reagan); virologist (Delaha); virologist (Stewart); professor and director of Live Stock Sanitary Service (Brueckner).

mice from each pool showed nervous symptoms, while the immunized mice from each strain appeared normal. This confirmed the identity of each virus used in this experiment.

Four mice were injected intracerebrally with the rabies pool, and at the end of six days these mice were paralyzed. Upon examination of these mouse brains using Seller's [10] stain, numerous Negri bodies were observed. This confirmed the rabies pool to contain the rabies virus.

TABLE 2—Response of Swiss Albino Mice Inoculated Intracerebrally and 10-day Embryonating Eggs Inoculated with Brain Suspension from Turtles for Each Group (Turtles Destroyed After a 21-day Observation Period

Virus strain	Mice exposed (No.)	Mice showing central nervous system involvement (No.)
Bunyamwera	4	0
Bwamba	4	0
Zika	4	0
Ntaya	4	0
Wyeomyia	4	0
Uganda "S"	4	0
Ilhéus	4	0
Anopheles "A"	4	0
Anopheles "B"	4	0
West Nile	4	0
Semliki Forest	4	0
Rabies	4	0

Virus strain	Eggs exposed (No.)	Eggs showing response (No.)
NDV (California)	4	0
NDV (Colorado)	4	0
NDV (Connecticut)	4	0
NDV (Delaware)	4	0
NDV (Kentucky)	4	0
NDV (Minnesota)	4	0

NDV—Newcastle disease virus.

The California strain egg-adapted thirty-fifth passage, Colorado strain egg-adapted twelfth passage, Connecticut strain egg-adapted twelfth passage, Kentucky strain egg-adapted twelfth passage, Delaware strain egg-adapted twelfth passage, and the Minnesota strain egg-adapted twelfth passage were confirmed by neutralization tests to be Newcastle disease virus in 10-day embryonating chicken eggs. These tests were conducted according to the procedure recommended by the Bureau of Animal Industry (*U.S.D.A. Bull.*, 1946).

EXPERIMENTAL

Seventy-two young green turtles *(Pseudemys elegans)* were divided into 18 groups of 4 turtles each. Into each group was administered 0.03 ml. of one of the above stated virus pool strains by intracerebral inoculation. These turtles were observed for central nervous system involvement over a period of twenty-one days (table 1). At the end of this period, the turtles were killed; the brains were removed aseptically, pooled, ground with alundum, and made into a 20 per cent suspension with sterile physiological saline.

Four 3-week-old Swiss albino mice were inoculated intracerebrally with 0.03 ml. of each of the pooled turtle brain suspensions except those from the Newcastle strains. The brain suspensions from the Newcastle turtles were injected into the allantoic cavity of 10-day embryonating eggs.

RESULTS AND DISCUSSION

The mice inoculated intracerebrally with the turtle brain suspensions were observed for fourteen days. At the end of this period, all of the mice of each group appeared normal (table 2).

The 10-day-old embryonating eggs inoculated with the Newcastle turtle brain suspensions appeared normal after an observation period of seven days; they were then discarded.

These results indicate that young green turtles *(Pseudemys elegans)* are not susceptible to these neurotropic viruses and that the viruses were not present in the turtle brains after the 21-day observation period.

SUMMARY

Seventy-two young green turtles *(Pseudemys elegans)* were placed into 18 groups of 4 turtles each. Mouse brain suspensions of Bunyamwera, Bwamba, Zika, Ntaya, Wyeomyia, Uganda "S", Ilhéus, Anopheles "A", Anopheles "B", West Nile, Semliki Forest, and rabies viruses, and allantoic fluid suspensions of the six strains of Newcastle disease viruses were injected intracerebrally into each group of turtles. At the end of twenty-one days these turtles appeared normal. The brains of the turtles were removed from each group and all but the Newcastle groups were inoculated intracerebrally into mice. At the end of fourteen days all the mice appeared normal. The six Newcastle groups each were injected into 10-day-old embryonating eggs and after seven days all of the embryonating eggs were normal. These results indicate that young green turtles are not susceptible to any of these neurotropic viruses.

References

[1] Smithburn, K. C., and Haddow, A. J.: Ntaya. A Hitherto Unknown Agent Isolated from Mosquitoes Collected in Uganda. Proc. Soc. Exptl. Biol. and Med., 77, (1951): 130-133.

[2] Garcia-Roca, Manual: The Isolation of Three Neurotropic Viruses from Forest Mosquitoes in Eastern Columbio. J. Infect. Dis., 75, (1946): 160-169.

[3] Smithburn, K. C., Mahaffy, A. F., and Paul, J. H.: Bwamba Fever and Its Causative Virus. Am. J. Trop. Med., *21*, (1941): 75-90.

[4] Smithburn, K. C., and Haddow, A. J.: Semliki Forest Virus. I. Isolation and Pathogenic Properties. J. Immunol., *49*, (1944): 141-157.

[5] Smithburn, K. C., Hughes, T. P., Burke, A. W., and Paul, J. H.: A Neurotropic Virus Isolated from the Blood of a Native of Uganda. Am. J. Trop. Med., *20*, (1940): 471-492.

[6] Smithburn, K. C., Haddow, A. J., and Mahaffy, A. F.: A Neurotopic Virus Isolated from Aedes Mosquitoes Caught in the Semliki Forest. Am. J. Trop. Med., *26*, (1946): 189-208.

[7] Laemmert, H. W., and Hughes, T. P.: The Virus of Ilhéus Encephalitis. Isolation, Serological Specificity and Transmission. J. Immunol., *55*, (1947): 61-67.

[8] Rivers, T. M.: Viral and Rickettsial Infections of Man. 2nd ed. J. P. Lippincott Co., Philadelphia, (1952): 253.

[9] Webster, L. T., and Dawson, J. R.: Early Diagnosis of Rabies by Mouse Inoculation. Measurement of Humoral Immunity to Rabies by Mouse Protection Test. Proc. Soc. Exptl. Biol. and Med., *32*, (1935): 570-573.

[10] Sellers, F. F.: A New Method for Staining Negri Bodies of Rabies. Am. J. Pub. Health, *17*, (1932): 1080-1081.

An Authorized Binding

for

American Journal of Veterinary Research

Arrangements have been made by American Veterinary Medical Association for subscribers to have their journals bound into distinctively designed books.

Four issues, January through December, bound in best grade blue washable buckram, imprinted with your name on cover, cost but $3.30 per volume.

Bound journals serve as an immediate reference for research and information. Properly displayed, they create a psychological impact, implying the time and effort spent to keep up-to-date on the most modern techniques and treatments.

Ship journals parcel post. Within thirty days after receipt, bound volumes will be shipped prepaid anywhere in the U.S.A. Full remittance must accompany order.

PUBLISHERS' AUTHORIZED BINDERY SERVICE

Binders of all Journals

308 West Randolph Street Chicago 6, Illinois

COPYRIGHT 1954 BY AMERICAN VETERINARY MEDICAL ASSOCIATION

Printed in the U.S.A